Development of Controlled Release Parenteral formulation of an antiplatelet drug Eptifibatide.

Summary for the degree of

Doctorate of Philosophy (Ph. D)

In

Pharmaceutical Sciences

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2023

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1. INTRODUCTION

Acute Coronary Syndrome

Acute coronary syndromes (ACS) is a broader term doctors use to refer to three types of cardiovascular conditions that affect millions of people each year, i.e., two types of myocardial infarction (STEMI & NSTEMI), and unstable angina. All these conditions arise due to thrombus formation in coronary arteries leading to decreased blood flow and thus reduced oxygen supply to heart muscles, resulting in ischemia and then infarction of that part. ACS is always symptomatic.[1][2][3]

The treatment used in ACS

There are two treatment options available for ACS. The patient is either put on medicines, or they are suggested to undergo a surgical procedure depending upon the conditions of the patient. Treatment with medication basically involves options that try to minimize or reverse either of the coagulation cascade steps that occur during the coagulation process.

Surgical procedures involve Coronary artery bypass surgery (CABG), here a new pathway is created to restore the blood supply to the affected part of the heart, by using a portion of the saphenous vein present in the leg and stitching it to the heart blood vessels.

Other surgical procedures might involve the placement of metal or biodegradable stents inside the affected blood vessel to widen the lumen and restore the blood supply.

Percutaneous Coronary Intervention (PCI)

It's a surgical procedure that might involve placing a stent or CABG. It is opted for in emergency situations to either access the root cause of ACS or to provide treatment. It improves outcomes and reduces the chances of morbidity and mortality. The initial treatment for all ACS includes the use of oral as well as parenteral anticoagulants and antiplatelet therapy. The American Heart Association (AHA) recommends an emergent catheterization and percutaneous intervention (PCI) for STEMI with a door-to-the-procedure start time of fewer than 90 minutes. [4] Cases where PCI can't to opted for, they are taken for CABG and or managed medically. Usually, such patients are put on parenteral anticoagulants from the time they are admitted and continued till the time they are discharged from the hospital.[5] Since these medicines prevent coagulation, they need to be aborted for some time before any surgical intervention as they might cause bleeding risk. Preference is given to antiplatelet drugs like Eptifibatide which are more specific and have rapid reversibility.[6] For maximum patient compliance and from a safety point of view these medications should have rapid onset and offset action and should not need continuous monitoring. Post-hospitalization these patients are put on anticoagulation therapy for longer periods of time. Adherence to this treatment is a task as there is always a risk of missing a dose or a repeated dose causing bleeding. Controlled-release treatment for

these agents might be helpful. Oral anticoagulants are considered as high alert medications according to the Institute of Safe Medication Practices (ISMP) because they have the potential for harm when used clinically.[7]

Deep Vein Thrombosis [8]

However, there are certain situations where clots are formed inside blood vessels without any injury, and they don't dissolve this can be critical and needs immediate medical attention. Thrombosis may happen in veins termed as venous thrombosis or it may happen in arteries termed as arterial thrombosis. Venous thrombosis at times is called deep vein thrombosis (DVT) which causes the formation of a blood clot in the affected part of the body, while arterial thrombosis disturbs the blood supply to a particular part of the body causing damage due to ischemia and necrosis. DVT is usually formed in deep veins of the legs such as the femoral vein. DVT can be life-threatening if the clot moves and travel inside and gets lodged in the pulmonary artery that supplies blood to the lungs. This is termed a pulmonary embolism (PE). DVT and PE are called as venous thromboembolism (VTE) and fall under cardiovascular disease. VTE mostly manifests as DVT only and PE.

Treatment for DVT [9]

Treatment for DVT depends on the site and the symptoms involved. Therapy with anticoagulants is a typical treatment. Treatment does involve continuous monitoring for bleeding episodes. As mentioned, treatment depends on the location of thrombus formation, so in the case of isolated distal DVT, treatment with ultrasound surveillance is used instead of anticoagulants. [10] Patients with proximal DVT are put on anticoagulants for four to six weeks [11] minimum anticoagulation treatment for three months is essential. [10]

Anticoagulants vs Antiplatelet drugs

Antiplatelet and anticoagulant drugs as the name suggests work at different sites for the common goal of preventing blood clots. Since platelets are mainly involved in the initial steps of atherosclerosis antiplatelets are prescribed in those patients. Whereas anticoagulants are used in patients with atrial fibrillation, and venous thromboembolism. Both anticoagulants and antiplatelets can be used together in certain clinical settings however there is always a risk of bleeding associated with combined therapy. Careful assessment and balance are needed before initiating the treatments. The clinical condition that needs more intensive antithrombotic therapy and involves long-term treatment then both anticoagulant and antiplatelet can be combined. Both classes of drugs can also be combined temporarily like after coronary intervention in patients with atrial fibrillation.

Antiplatelets

As the name suggests these medications prevent the activity of platelets. There are two types of antiplatelet drugs depending upon the route of administration. Orally administered and parenterally administered antiplatelet drugs. Antiplatelets are also classified based on the mechanism of action, please refer to the table below for details on all types of antiplatelets drugs.

Table No. 1 Details of oral and parenteral antiplatelet drugs

Sr. No.	Antiplatelet Drug	Therapeutic Category	Mechanism of Action	Treatment duration
	ORALLY ADMINISTERED			
1	Aspirin	Nonsteroidal anti-inflammatory drugs (NSAIDs)	Inhibit platelet thromboxane A_2 synthesis and reduce thrombus formation damaged arterial wall.	6 months (DAPT)
2	Clopidogrel	Platelet aggregation inhibitors	Inhibits the binding of adenosine diphosphate (ADP) to its platelet P2Y12 receptor and prevents activation of the glycoprotein GPIIb/IIIa complex.	12 months (DAPT)
3	Ticagrelor	Platelet aggregation inhibitors	Reversibly interacting with the platelet P_2Y_{12} adenosine diphosphate (ADP) receptor to prevent signal transduction.	12 months
4	Ticlopidine	Platelet aggregation inhibitors	Inhibit binding of ADP to platelet receptors and prevent activation of glycoprotein IIb/IIIa complex.	1-2 months
5	Prasugrel	Platelet aggregation inhibitors	Inhibit platelets activation by binding ADP P2Y12 receptor.	Up to 1 year
6	Pentoxifylline	Hemorheological agents	It reduces the viscosity of blood by decreasing erythrocyte aggregation and stimulates fibrinolysis.	6 months
7	Vorapaxar	Thrombin receptor antagonist (TRA)	Inhibits thrombin receptor-activating peptide (TRAP) induced platelet aggregation.	30 -60 days
8	Dabigatran	direct thrombin inhibitors (DTIs)	Inhibits thrombin and thus the formation of fibrin	Up to 35 days
9	Dipyridamole	Nucleoside transport inhibitor phosphodiesterase type 3	Inhibits platelet cyclic nucleotide phosphodiesterase	3- 6 months
10	Cilostazol	PDE3 inhibitor	Inhibits the platelet aggregation induced by collagen, 5'-adenosine diphosphate (ADP), epinephrine, and arachidonic acid.	1-3 months
	PARENTERALLY ADMINISTERED			
11	Cangrelor	P2Y12 platelet inhibitor	Direct $P2Y_{12}$ platelet receptor inhibitor that blocks ADP-induced platelet activation and aggregation.	Till the time the patient is discharged.
12	Abciximab	Glycoprotein platelet inhibitors	Inhibits platelet aggregation by preventing the binding of fibrinogen, von Willebrand factor, to GPIIb/IIIa receptor.	Till the time the patient is discharged.
13	Eptifibatide	Glycoprotein platelet inhibitors	Inhibits platelet aggregation by preventing the binding of fibrinogen, von Willebrand factor, to GPIIb/IIIa receptor.	Till the time the patient is discharged.
14	Tirofiban	Glycoprotein platelet inhibitors	Inhibits platelet aggregation by preventing the binding of fibrinogen, von Willebrand factor, to GPIIb/IIIa receptor.	Till the time the patient is discharged.

DAPT: Dual antiplatelet therapy

Eptifibatide

Eptifibatide is an antiplatelet drug that inhibits platelets' glycoprotein IIb/IIIa receptors and exhibits rapid reversibility. It is derived from a protein found in the venom of southeastern pygmy rattlesnake. It has a highly specific binding to the Gp IIb/IIIa receptor because of the structural resemblance of the KGD (Lys-Gly-Asp) sequence.[6] It is indicated for the treatment in ACS for medical management of unstable angina (UA) and non-ST elevation myocardial infarction. It has also been approved in patients undergoing PCI with intracoronary stenting. The primary pathological mechanism involved in ACS is the formation of thrombosis which is mediated by platelets.[12]The cross-linking of the receptor glycoprotein (GP) IIb-IIIa on adjacent platelets by adhesives protein fibrinogen is the final common pathway. So, inhibiting this step is very crucial and can be more specific. This has demonstrated unequivocal clinical benefits in ACS. Despite being treated by glycoprotein (GP) IIb-IIIa inhibitors there are situations where there is a need to perform emergency CABG. This increases the risk of bleeding and further complicates the treatment. This effect is basically due to the prolonged action of the glycoprotein (GP) IIb-IIIa inhibitors and initiation of the surgical procedure within a few hours of terminating the treatment. There are three glycoproteins (GP) IIb-IIIa inhibitors Abciximab, Eptifibatide, and Tirofiban. approved by U.S. Food and Drug Administration. Abciximab shows prolonged antiplatelet action and is associated with bleeding problems. Eptifibatide is a peptide and exhibits immediate reversible action. Whereas Tirofiban HCl is a nonpeptide that also exhibits immediate reversible action. Therefore, when there is a need for CABG, Eptifibatide and Tirofiban are preferred over Abciximab as they are associated with fewer bleeding episodes.

Properties of intravenous GP IIb-IIIa inhibitors[13]

Abciximab It is an antibody fragment, with a large size of 48 kilodaltons and has a rapid onset of action with slow reversibility of antiplatelet effect (> 12 hours) thus might increase the risk of bleeding, particularly in patients requiring CABG shortly after termination of an infusion. It does not have specificity for GP IIb-IIIa receptors and binds to the closely related vitronectin receptor.

Tirofiban is a nonpeptide, with a small size (< 1kda), and has a rapid onset of action. It shows rapid reversibility of 4-8 hours thus preferred in patients needing CABG immediately after termination of treatment and has specificity to receptors.

Eptifibatide is a peptide, with a small size (< 1kda) and has rapid onset of action, rapid reversibility 4 hours preferred in patients needing CABG immediately after termination of treatment and has specificity to receptors.

Biodegradable Polymer based Microspheres for Controlled drug delivery.

With improved understanding and technological updates in biotechnology, many proteins and peptides have been invented, which are being used to treat diseases that are incurable or chronic. The market for proteins and peptides is growing at an ace speed and would surpass the market of smaller molecules in the near future. [14] Despite this very few proteins and peptides reach the market. This may be due to the need for a specialized drug delivery system with a sophisticated manufacturing process, as oral delivery is ineffective due to poor stability in gastric fluid. The parenteral route using a hypodermic needle and syringes remains the best and the most preferred route for administering these therapeutic proteins. However, formulating a drug delivery system for parenteral delivery is a big challenge[15] The bioavailability of proteins and peptides is affected by, systemic proteases, structural changes, short half-life, opsonization, and damage to labile side groups.[14] [16] Hence novel approaches are explored to successfully deliver proteins and peptides parenterally. Different delivery systems like liposomes, lipid vesicles, lipid emulsions, implants, and microspheres have been developed to deliver therapeutic proteins. Each one of them has its advantages and complexity. Amongst all these, microspheres have received considerable attention and are one of the hot research topics. Microspheres give a protective enclosure for therapeutic proteins, thus preventing exposure to external hydrolytic and enzymatic stress. Long-acting microspheres would achieve better patient compliance as patients will have few injection pricks and sometimes even self-administration is possible without the need for the patient to visit the health care provider. Encapsulation of proteins by microspheres would help deliver them in a controlled manner. Microspheres provide unique advantages and overcome the problems of delivering proteins and peptides by conventional injectables. With the availability of biodegradable polymers used to formulate these microspheres, more interest has been generated and has further boosted research on these novel drug delivery systems.

However, despite having so many advantages it also has so many challenges involved. The major drawback involved is in the cost of the polymers and availability of the right grade of polymer is crucial and challenging. The higher polymer cost is translated into high product cost, thus even though essential and patient-friendly, not all patients can afford these novel drug delivery systems. Of the other critical challenges involved in development of these biodegradable polymer-based microspheres, low drug entrapment, unwanted burst release, stability of the entrapped drug are more crucial and challenging which has over reduced the application of these wonderful drug delivery system.

2. AIM AND SCOPE OF WORK

Rationale

The patients with ACS are often put on an antiplatelet drug immediately after admission and this treatment is continued till the time the patient is discharged. Temporary cessation of this therapy is done if the patient is supposed to undergo PCI. These patients are advised to have oral anticoagulants for a longer period more than a year. There is an increasing trend to prescribe multiple anticoagulants to the patients due to in adequacy of available oral treatments. Dual antiplatelet therapy increases the risk of bleeding two to three anticoagulants work in synergy and needs continuous monitoring. The currently marketed formulation of Eptifibatide is a conventional injection that needs to be administered by iv infusion which causes the patient to be confined to bed. Single treatment with Eptifibatide has shown better efficacy and have reduced incidences of myocardial infarctions. In developing countries where resources are less and are burdened with more population confining the patient to bed just for administering a particular drug is not a good idea. Also, if the patient is continuously confined to his bed there is a higher chance for him to develop DVT. Single therapy with Eptifibatide have shown better therapeutic efficacy and reduced incidences. Considering all these scenarios administering Eptifibatide in the form of depot injection will serve the purpose. It will release the drug for a prolonged period after a single injection thus not requiring an infusion line and the patient is not confined to the bed. The developed delivery system will provide a therapeutic advantage over oral therapy thus justifying the need to develop.

Thus, there is a need to work in this direction and address critical challenges faced while formulating biodegradable polymer-based microspheres. The present research work will try to address the common issues of drug entrapment, drug stability, and in vitro release. This research work will also evaluate formulating alternate dosage forms such as implants and in situ implants and nanoparticles and compare these against the developed microspheres.

Objectives

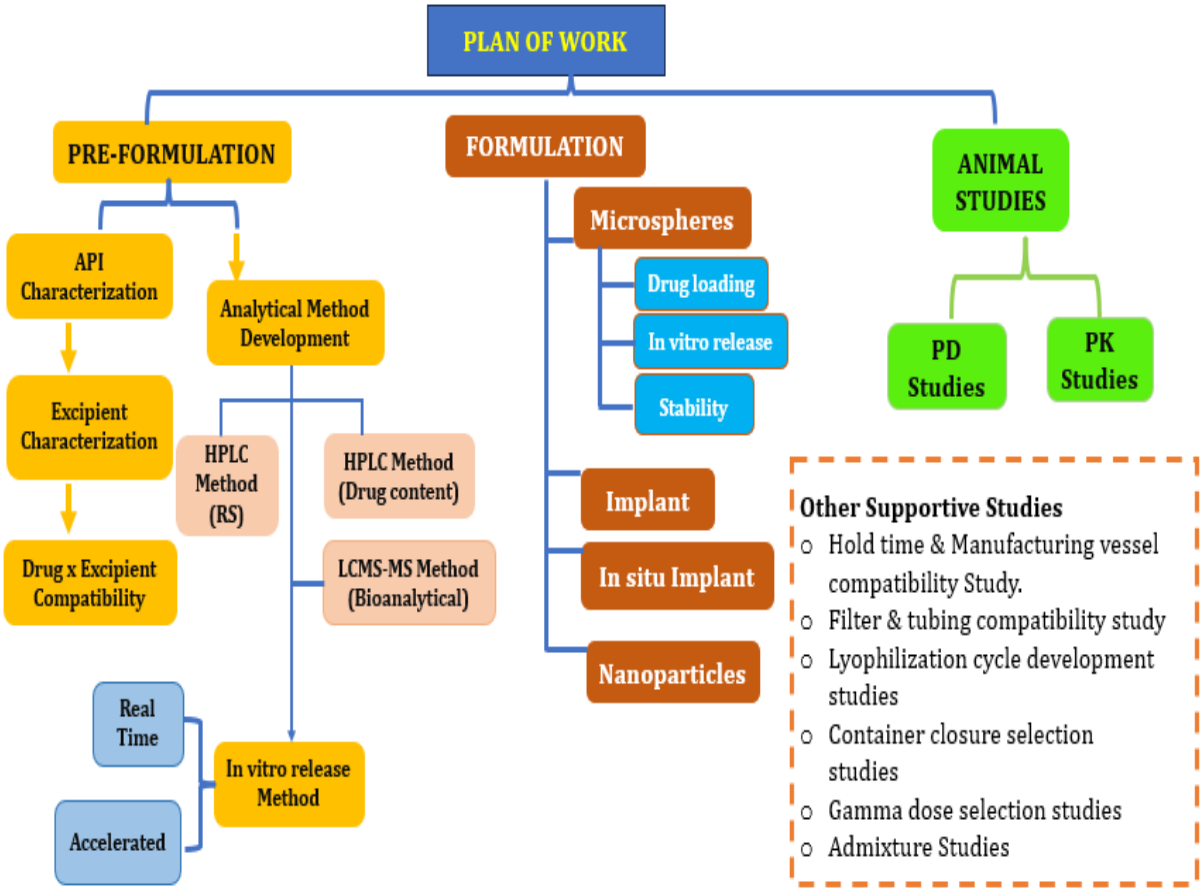
The main objectives of this research project are mentioned below:

A. To develop biodegradable polymer-based microspheres and address the existing problems associated with them like drug entrapment, drug stability, and dissolution profile.
B. To develop an implant or in situ implant using a biodegradable polymer.
C. To develop a formulation using a biodegradable polymer with polymer PEGylation and or use of Phospholipids which would help target the drug.
D. To explore the feasibility of using a controlled-release formulation of Eptifibatide in deep vein thrombosis.

Plan of work

The development work was carried out keeping in mind the following things.

- The manufacturing method used must be simple and easy to scale up.
- The process should involve the use of less amount of organic solvents/ green solvents.
- Use of drug overages for desired drug entrapment to be avoided.
- The process should yield a product with low bioburden, so terminal sterilization if done using gamma irradiation can be done at a lower dose.



3. ANALYTICAL METHOD DEVELOPMENT

○ UV method development

The UV-visible Spectrophotometric method was basically used as a base to start the initial work and to finally develop and then shift to HPLC method.[17] The assay of developed batches was performed by High-performance Liquid Chromatography (Shimadzu, Japan) and then validated as per ICH guidelines for specificity, precision, linearity, range, accuracy, ruggedness, and robustness. Eptifibatide is slightly soluble in water hence for initial method development, purified water was used as a solvent for standard preparation.

○ High-performance Liquid Chromatography (HPLC) method development

For accurate analysis of the developed batches, a High-performance liquid chromatography method was developed. Literature based method was opted for and further optimized to get the desired peak without interference and at a short retention time.[18]

Preparation of standard stock solution

About 10 mg of Eptifibatide was dissolved in 20 ml of purified water in a volumetric flask, 4 ml of this solution was further diluted to 10 ml with mobile phase to give a solution (200 µg/ml), i.e., 200 ppm. This solution was then filtered through a syringe filter (0.45 µm).

Preparation of mobile phase

The mobile phase consisted of a mixture of Acetonitrile (ACN) and 50 mM buffer solution of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) in a 1:3 ratio (25:75 v/v) with the pH of the buffer adjusted to 2.2 using orthophosphoric acid (OPA). 50 mM buffer solution of sodium dihydrogen orthophosphate was prepared by dissolving about 7.8 g of sodium hydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_20$) in a small amount of purified water, and the volume was made up to 1000 ml. (Observed pH of this buffer was 4.48). About 750 ml of this buffer was taken in a measuring cylinder and to this 250 mL acetonitrile was added. The pH of this buffer was then adjusted to 2.2 with Orthophosphoric Acid (OPA). Orthophosphoric acid was directly used without dilution, the amount of OPA required for pH adjustment was 7-8 ml. The mobile phase was then filtered through 0.2 µm filter paper and later degassed for 10-15 minutes by sonication. The buffer was then stored in clean 5 litres schott glass bottle until further use.

Preparation of extraction buffer (Acetate Buffer pH 4.1)

Acetate buffer is used to extract the drug out of the microspheres. About 5 g of sodium hydroxide was dissolved in a small amount of purified water, to this 30 ml glacial acetic acid

was added and the solution was then diluted to 1000 ml with purified water. The pH of the buffer was found to be 4.1.

Table No. 2 HPLC column and chromatographic conditions for drug content

Sr. No.	Parameters	Details
1	Column	Thermo hypersil BDS column C18 (250 mm x 4.6 mm) I.D.5 µ particle size
2	Flow rate	1 ml/min
3	Detector	Photodiode array (PDA)
4	Wavelength	220 nm
5	Injection volume	20 µl
6	Sample temperature	25° C
7	Run time	6 minutes.
8	Mobile Phase	Mixture of ACN: 50 mM sodium dihydrogen ortho phosphate with pH adjusted to 2.2 with OPA (25:75 v/v) in isocratic mode

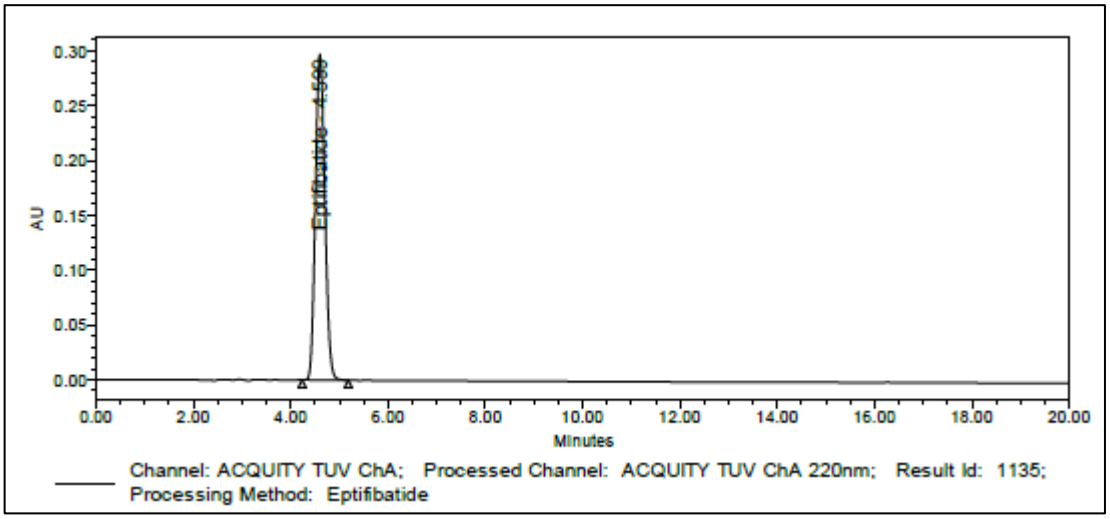


Figure No. 1 HPLC chromatogram for Standard solution of Eptifibatide.

Validation of the developed HPLC method

As per the recommendation of ICH [19] validation studies were performed to evaluate the suitability of the analytical method used for the assay of Eptifibatide. The method was validated for the following validation parameters.

Table No. 3 Validation parameters as per ICH guidelines

Sr. No.	Validation parameters
1	Specificity
2	Linearity
3	Range
4	Accuracy
5	Precision (Repeatability, intermediate precision)
6	Robustness
7	System suitability
8	Solution state stability

- **Bioanalytical method development**

The developed formulation will be evaluated for its pharmacokinetic and pharmacodynamic characteristics, hence there is a need to have an analytical method which will help evaluate the drug concentration in animal plasma. A literature reference method was used to evaluate the drug concentration in animal plasma.[20]

Table No. 4 Column and chromatography conditions for bioanalytical method

Sr. No.	Parameters	Details
1	Column	Venusil ASB-C18 column (150× 4.6 mm) Internal Diameter of 5 µm.
2	Security guard	Guard C18 guard column (4 x 3.0 mm)
3	Flow rate	0.5 ± 0.1 ml/min with the isocratic mobile phase
4	Detector	API 3200 triple-quadrupole mass spectrometer
5	Ion spray voltage	+5.5 kV
6	Injection volume	20 µl/min
7	Heater gas temperature	500° C
8	Mobile Phase	Acetonitrile: 5 mM Ammonium Acetate: Acetic Acid (30:70:01, v/v/v)

Collection of blood sample and separation of Plasma

1 ml of blood sample was collected in a test tube containing Ethylenediaminetetraacetic acid (EDTA). This was then mixed and vortexed for 15 minutes at 3000 rpm. The supernatant was collected as a plasma sample. About 500 µl of methanol was added to 100 µl of the above plasma samples. This was vortex-mixed for 5 min, followed by centrifugation at 15000 rpm for 5 min at 4° C. The supernatant was transferred to a clean glass tube and evaporated to dryness at 40° C under a stream of nitrogen in the TurboVap evaporator. The residue was reconstituted in 200 µl of the mobile phase and vortex-mixed for 1 min. A 20 µl aliquot of the reconstituted extract was injected for LC-MS/MS analysis.

Preparation of blank plasma sample was done by spiking 90 µl of plasma with 10 µl of methanol and the same process described above was followed.

Preparation of Standard solution

About 10 mg of Eptifibatide was dissolved in 20 ml of purified water in a volumetric flask, 4 ml of this solution was further diluted to 10 ml with mobile phase that give a solution of 200 µg/ml, i.e., 200 ppm. This solution was then filtered through a syringe filter of 0.45 µm. The stability of the drug in rat plasma was also evaluated for about 14 days.

Drug stability in rat plasma

The stability of the drug in rat plasma was evaluated, where the drug-containing plasma samples were placed in the deep freezer, 2-8° C and at room temperature for up to 14 days.

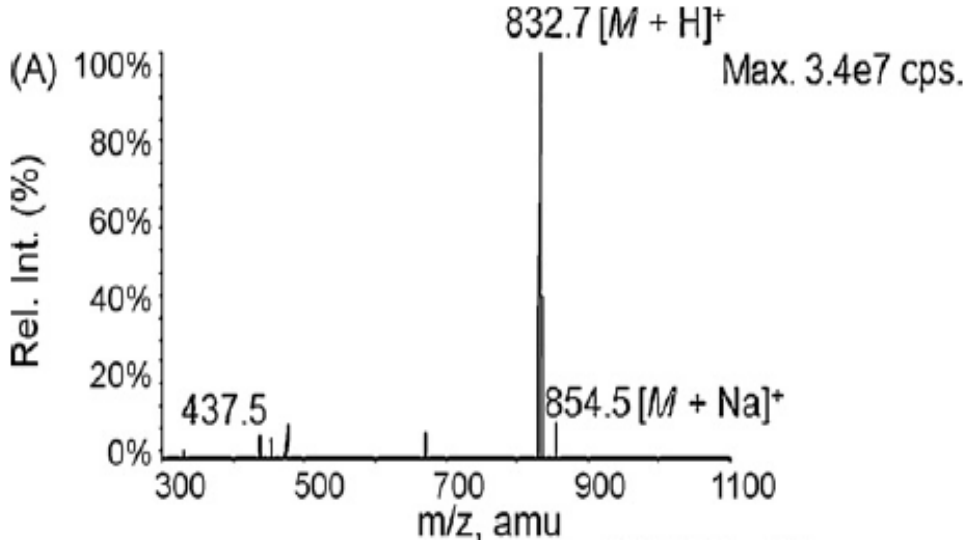


Figure No. 2 Full scan mass spectra for Eptifibatide using LCMS-MS

- **Related substance method**

Related substances help identify the degradants present in the raw material or produced during the processing of the formulation. This method also helps to identify the stability of the developed formulation by identifying degradants formed on storage at different conditions. A literature reference method was used for identifying the degradants of the developed formulation.[21]

Table No. 5 HPLC column and chromatographic conditions for related substances

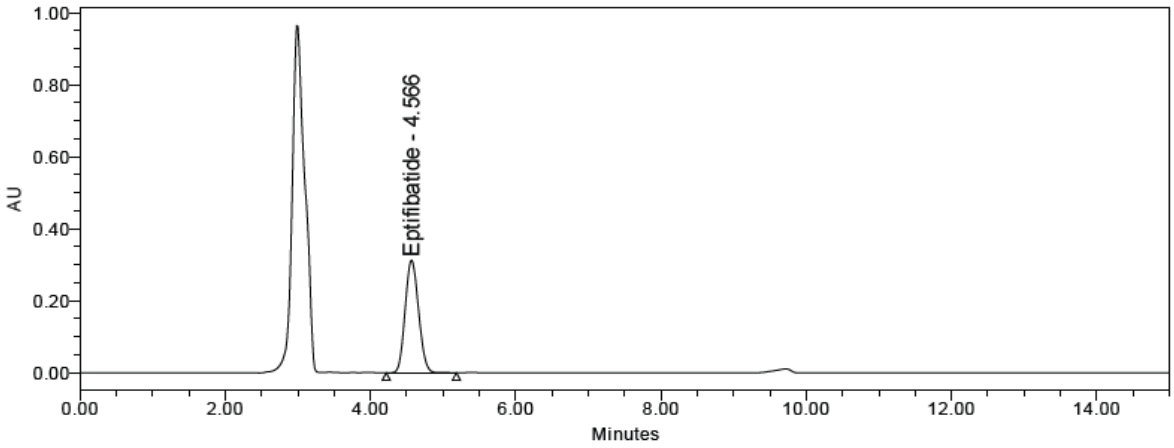
Sr. No.	Parameters	Details
1	Column	YMC-Pack-ODS-AQ (250 x 4.6 mm, 5 µm) 200A°
2	Flow rate	0.8 ± 0.1 ml/min
3	Detector	UV Detector
4	Wavelength of measurement	220 nm
5	Injection volume	40 µl
6	Column temperature	55° C ± 2° C
7	Run time	85 min

Mobile Phase

1. For part A, about 0.8 g of Heptane-1-sulphonic acid sodium salt was taken in 950 ml water, this solution was filtered through a 0.45 µm filter.
2. About 50 ml buffer of step 1 was taken in a 100 ml beaker, to this 0.6 g of Trifluoracetic acid was added.
3. The buffer of step 2 was then transferred to a 1000 ml glass bottle, and the beaker was rinsed twice. The buffer of step 1 was then transferred to the same 1000 ml glass bottle and 50 ml

of Acetonitrile was added to it. The solution was sonicated for a few minutes and the pH was adjusted to 3.7 ± 0.05 with TEA.

4. For part B, about 0.8 g of Heptane-1-sulphonic acid sodium salt was dissolved in 200 ml water and filtered through a 0.45 µm filter.
5. About 50 ml of the buffer of step 4 was taken in a 100 ml glass beaker and 0.6 g of Trifluoracetic acid was added to it. This was then transferred to a 1000 ml glass bottle.
6. About 800 ml of Acetonitrile was added to the buffer of step 5 and sonicated for a few minutes and the pH was adjusted to 3.7± 0.05 with TEA.



Processed Channel Descr.: ACQUITY TUV ChA 220nm

	Processed Channel Descr.	Peak Name	RT	Area	% Area	Height
1	ACQUITY TUV ChA 220nm	Eptifibatide	4.566	4006764	100.00	312812

Figure No. 3 HPLC chromatogram for Standard chromatogram for Eptifibatide for related substance method development

4. CHARACTERISATION OF API

The research molecule Eptifibatide was received as a gift sample along with a certificate of analysis (COA) from Ms. USV Pharmaceutical Pvt Ltd, Chembur, Mumbai. We are deeply thankful to Ms. USV Pharmaceuticals for understanding our need and accepting our request to provide a gift sample.

Table No. 6 Batch details of Eptifibatide

Name	Supplied by	Batch No.	Mfg. Date	Retest Date
Eptifibatide	USV Pharmaceuticals Pvt Ltd.	RD000X	Feb 2018	Jan 2023

Eptifibatide is not official in any pharmacopeia. The batch of Eptifibatide was analyzed for the test mentioned against the COA, only selected tests were carried out. The list of tests and the results obtained are discussed in the Results and discussion section.

Table No. 7 Analytical data for Eptifibatide

Sr. No.	Test		Specification	Results (COA)	Results obtained
1	Description		White powder	White to off white powder	White to off white powder
2	Clarity of solution		The solution in water and in 1% acetic acid at a concentration of 1 mg/ml gives a clear solution	Complies	Complies
3	Identification				
	3.1	HPLC	Should match STD peak.	Complies	Complies
	3.2	IR	Should match STD graph	Complies	Complies
4	Assay by HPLC (Anhydrous and aceticacid-free substance basis)		95.0% to 105.0%	101.1%	102.1%
5	DSC		For information purpose	Not done	49.67 °C
6	Bacterial Endotoxin		NMT 0.5 EU/mg	Less than 0.5 EU/mg	Complies
7	Microbial Limit test				
8	Total viable aerobic count				
	TAMC		NMT 1000 CFU/g	Less than 200	Complies
	TYMC		NMT 100 CFU/g	Less than 20	Complies
	Pathogenic and selected sort of microorganisms				
	Escherichia coli		Should absent / 50 mg	Absent / 50 mg	Absent
	Slamonella		Must be absent / 50 mg	Absent/ 50 mg	Absent
	Pseudomonas aeruginosa		Must be absent / 50 mg	Absent/ 50 mg	Absent
	Staphylococcus aureus		Must be absent / 50 mg	Absent/ 50 mg	Absent

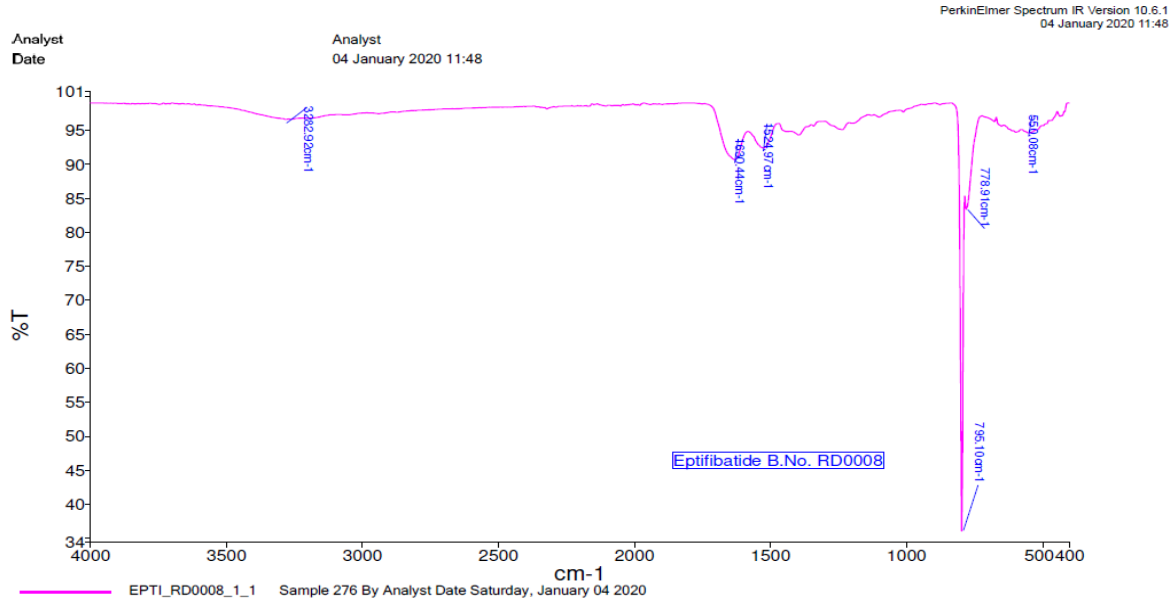


Figure No. 4 IR Graph for Eptifibatide

Table No. 8 Functional group relation for Eptifibatide in IR Graph.

Sr. No.	Band (cm^{-1})	Assignment
1	3282	-OH stretching
2	1630	-NH bending
3	1525	N-O stretching
4	795	C=C binding

The received lot of Eptifibatide was found to be suitable and thus was used in our research project.

5. CHARACTERISATION OF EXCIPIENTS

All the excipients used were of standard grade and within shelf life, majority of the excipients were approved and used based on the Certificate of analysis (COA) provided by the vendor, and only critical excipients like biodegradable polymers were characterized thoroughly.

- **Biodegradable polymer**

All the different lots of biodegradable polymers were purchased from Evonik India Ltd. Typical details for Biodegradable Polymers are mentioned in the table below.

Table No. 9 List of biodegradable polymers.

Supplier	Brand Name	Polymer Type	Ratio (LA:GA)	End Group	Lot No.	IV(dl/g)
Evonik India Pvt Ltd	Resomer RG752H	Copolymer	75:25	Acid terminated	D150500554	0.17
	Resomer RG752H	Copolymer	75:25	Acid terminated	D170800543	0.20
	Resomer RG502H	Copolymer	50:50	Acid terminated	D200100502	0.21
	Resomer RG503H	Copolymer	50:50	Acid terminated	D180900527	0.38
	Resomer RG504H	Copolymer	50:50	Acid terminated	R130800529	0.53
	Resomer R202 H	Homo polymer	100:00	Acid terminated	R130500516	0.19
	Resomer R202 S	Homopolymer	100:00	Ester Terminated	D160600540	0.22

All the grades of biodegradable polymers were analysed for the following list of parameters.

Table No. 10 Characterisation tests for biodegradable polymers

Sr. No.	Test	The analytical method used
1	Co-monomer ratio	Proton NMR
2	Acid End group determination	C 13 NMR
3	Inherent viscosity	Viscometry
4	Glass transition temperature	Differential Scanning calorimetry.
5	Molecular weight	Gel permeation chromatography.

Table No. 11Analytical data for all the batches of biodegradable polymers.

Polymer	**RG752H**	**R202H**	**RG502H**	**RG503H**	**RG504H**	**R202S**
Batch No.	**D150500554**	**R130500516**	**D200100502**	**D180900527**	**R130800529**	**D160600540**
NMR	Yes	Yes	Yes	Yes	Yes	Yes
Comonomer Ratio						
DL Lactide	74.31	100	50.01	49.01	50.01	100
Glycolide	25.69	00	50.01	50.09	50.00	00
Residual Monomer Content						
DL Lactide	0.5	0.18	0.26	ND	1.02	0.01
Glycolide	0.002	ND	0.83	0.22	0.15	0.00
IV (dl/g)	0.17	0.19	0.21	0.38	0.53	0.22
Mol. Wt.	12523	13304	17306	30226	37301	14045
TG	34.33	41.42	42.91	42.61	44.35	48.2

The available grades of polymer complied as per the values mentioned in the COA and values obtained in house.

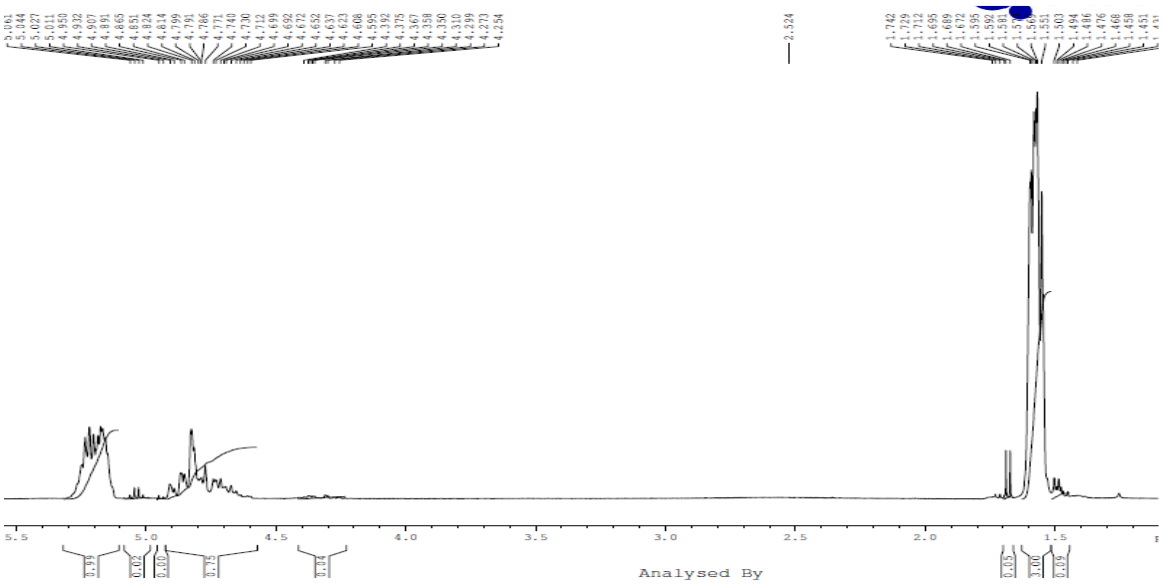


Figure No. 5 NMR of Resomer RG752H Batch No.D150500554

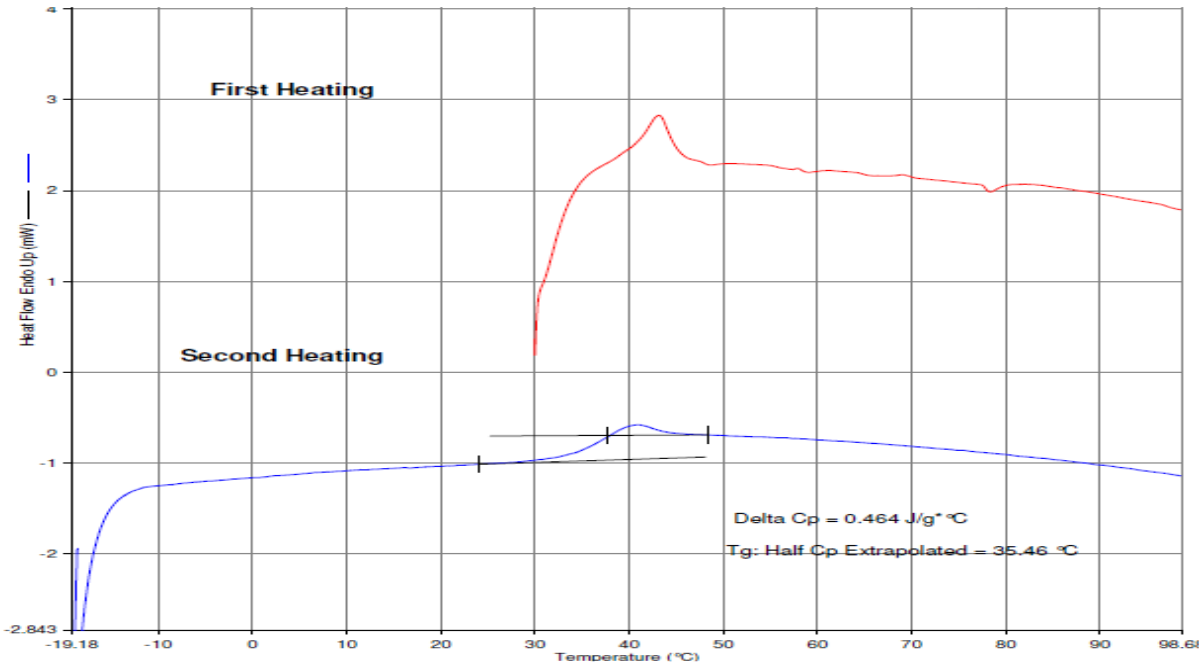


Figure No. 6 DSC Graph for Polymer, Resomer RG752H, Batch No.D150500554

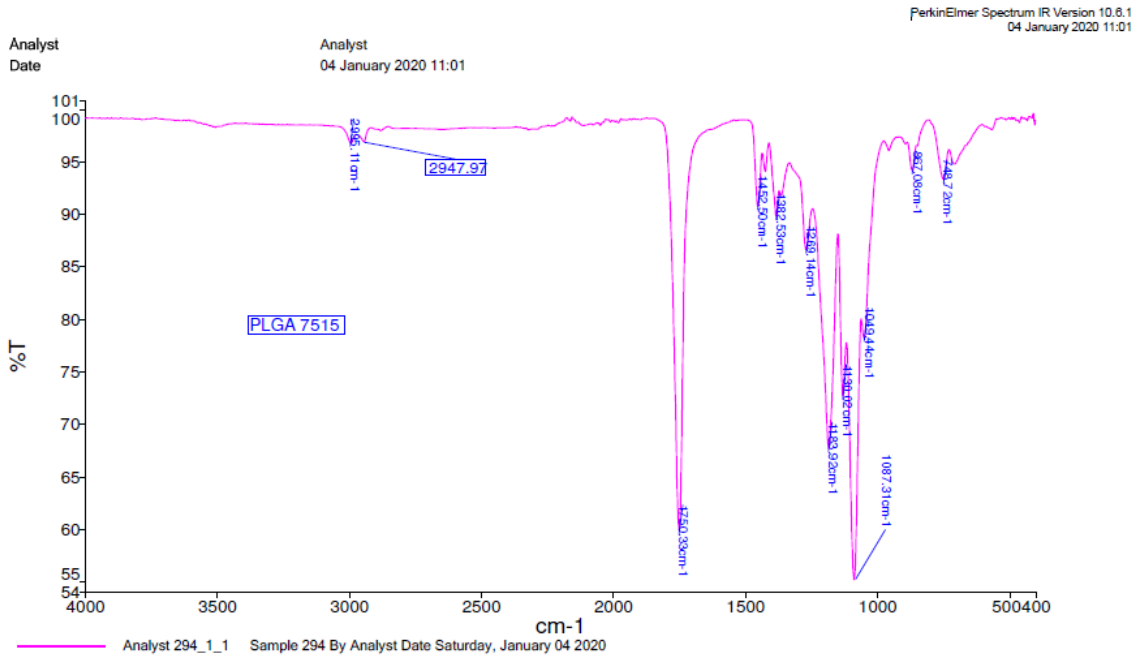


Figure No. 7 IR Graph of polymer

The presence of following NMR peaks as defined in the below table, indicates the type of polymer.

Table No. 12 NMR peak and respective functional group

Range	1.52 ppm	5.20 ppm	4.85 ppm
Function group	Methyl (CH_3)	Methine (CH)	Methylene (CH_2)
PLA Homo polymer	Peak present	Peak present	Peak Absent
PGA Homo polymer	Peak Absent	Peak Absent	Peak present
PLGA Copolymer	Peak present	Peak present	Peak present

6. DRUG EXCIPIENT COMPATIBILITY STUDIES

The basic intention of doing a drug excipient compatibility study is to identify well in advance any drug incompatibility issue which may arise due to the use of any excipient. A drug excipient compatibility study was conducted in accordance with ICH Q8 guidelines [22]where a mixture of drug and the prospective excipient were mixed in a ratio that will possibly appear in the final formulation.

Method

Both the drug and the excipient were weighed and then mixed uniformly; the said mixture was then filled in 5 ml Type I tubular glass vials supplied by Schott. The vials were closed with RTU bromobutyl rubber bungs and then sealed with aluminium flip-off seals. The glass vials were then subjected to stability for about one month at two conditions 30°C /65% RH and in cold room at 2-8° C. Samples were analysed for physical as well as chemical parameters.

- **Physical Parameters:** Appearance, should be similar to initial samples.
- **Chemical Parameters:** Drug content**,** Related Substances, and IR.

Table No. 13 Details of ratio used in drug excipient compatibility studies.

Sr. No.	Batch No.	Ingredients	Ratio
1	EP/88-A	Eptifibatide (E)	--
2	EP/88-B	PLGA Polymer	--
3	EP/88-C	PLA Polymer	
4	EP/88-D	D-Mannitol	--
5	EP/88-E	Carboxymethyl cellulose Sodium	--
6	EP/88-F	Trehalose	--
7	EP/88-G	Hydroxypropyl β Cyclodextrins	--
8	EP/88-H	Methoxy Polyethylene glycol (mPEG)	--
9	EP/88-I	Ditsteroyl phosphotidyl glycerol (DSPG –Sodium)	--
10	EP/88-J	Polyvinyl Alcohol (PVA)	--
11	EP/88-K	Eptifibaide + PLGA	1:10
12	EP/88-L	Eptifibatide + PLA	1:10
13	EP/88-M	Eptifibatide + D-Mannitol	1:1
14	EP/88-N	Eptifibatide + Sodium CMC	1:1
15	EP/88-O	Eptifibatide + Trehalose	1:1
16	EP/88-P	Eptifibatide + HPβCD	1:1
17	EP/88-Q	Eptifibatide + mPEG	1:1
18	EP/88-R	Eptifibatide + DSPG –Sodium	1:1
19	EP/88-S	Eptifibatide + PVA	1:1
20	EP/88-T	Eptifibatide+ PEG 400	1:1

The table below gives complete analytical data for samples charged on stability

Table: 14 Analytical data for Drug Excipient Compatibility study

Sr.	Batch No.	Ingredients	Ratio	Storage Condition	Assay	Total Imp.
1	EP/88-A	Eptifibatide (E)	NA	Initial	89.32%	1.45%
				30 °C/65 %RH 1 M	88.11%	1.56%
				2 to 8 °C 1 M	89.12%	1.48%
2	EP/88-K	E + PLGA	1:10	Initial	93.01%	2.01
				30 °C/65 %RH 1 M	92.11%	2.11
				2 to 8 °C 1 M	91.34%	2.01
3	EP/88-L	E + PLA	1:10	Initial	95.34%	2.11
				30 °C/65 %RH 1 M	94.34%	2.13
				2 to 8 °C 1 M	94.77%	2.11
4	EP/88-M	E + D-Mannitol	1:1	Initial	93.00%	2.01
				30 °C/65 %RH 1 M	92.14%	2.12
				2 to 8 °C 1 M	92.99%	1.98
5	EP/88-N	E + Sodium CMC	1:1	Initial	96.45%	2.11
				30 °C/65 %RH 1 M	94.67%	2.23
				2 to 8 °C 1 M	93.22%	2.10
6	EP/88-O	E + Trehalose	1:1	Initial	95.70%	1.88
				30 °C/65 %RH 1 M	94.89%	1.89
				2 to 8 °C 1 M	94.77%	1.87
7	EP/88-P	E + HP β CD	1:1	Initial	96.07%	1.76
				30 °C/65 %RH 1 M	96.78%	1.83
				2 to 8 °C 1 M	96.45%	1.79
8	EP/88-Q	E + mPEG	1:1	Initial	95.88%	2.10
				30 °C/65 %RH 1 M	95.44%	2.11
				2 to 8 °C 1 M	95.33%	1.99
9	EP/88-R	E + DSPG –Na.	1:1	Initial	95.34%	2.11
				30 °C/65 %RH 1 M	95.22%	2.01
				2 to 8 °C 1 M	95.15%	2.00
10	EP/88-S	E + PVA	1:1	Initial	94.55%	2.23
				30 °C/65 %RH 1 M	93.56%	2.40
				2 to 8 °C 1 M	94.34%	2.20
11	EP/88-T	E + PEG 400	1:1	Initial	95.43%	2.24
				30 °C/65 %RH 1 M	94.44%	2.23
				2 to 8 °C 1 M	95.21%	2.22
12	EP/49	MSP	1:1	Initial	97.12%	1.90
				30 °C/65 %RH 1 M	96.45%	1.93
				2 to 8 °C 1 M	97.22%	1.91
13	EP/70	IMPLANT	1:1	Initial	94.34%	2.23
				30 °C/65 %RH 1 M	93.45%	2.25
				2 to 8 °C 1 M	94.10%	2.21
14	EP/55	MSP (mPEG)	1:1	Initial	95.22%	1.85
				30 °C/65 %RH 1 M	94.35%	1.89
				2 to 8 °C 1 M	94.34%	1.78

All the combinations at all different ratios were found to be stable with no significant changes in both physical and chemical attributes. Thus, no incompatibility was seen with the proposed excipients used. The mixtures had the assay values within acceptable range (90-110%). The related substances observed for all the mixtures were within acceptable range NMT 2.5% Temperature of 30° C was chosen for compatibility studies as the glass transition temperature of the polymer is in the range of 35-40° C.

7. FORMULATION DEVELOPMENT OF MICROSPHERES

The development work was carried out keeping the following things in mind.

- The manufacturing method used must be simple and easy to scale up.
- The process should involve the use of less amount of organic solvents/ green solvents.
- Use of drug overages for desired drug entrapment to be avoided.
- The process should yield a product with low bioburden, if radiation sterilization (Gamma Irradiation) is used then approaches to reduce the effect or use of a lower dose.

Development of Eptifibatide Microspheres

- **Formulation of Microspheres using single emulsion solvent evaporation method here Dimethyl formamide was used as drug solvent**

To begin with, the quantitative composition for the marketed product Lupron depot was used as a prototype. The ratio of the drug to polymer was titrated to achieve desirable drug loading (>85% w/w). Here Eptifibatide microspheres were formulated using a single emulsion solvent evaporation method. Dimethylformamide was used as solvent to dissolve the drug, and dichloromethane was used to dissolve the polymer. The amount of DCM and DMFA was kept as minimum as possible, i.e., to keep both the polymer and the drug in a completely solubilised state and at the same time the solution obtained is pourable. Polyvinyl alcohol was used as surfactant at concentration of 0.25% w/w. The ratio of internal phase to external phase was kept very high to have good drug loading. The speed during homogenisation was kept at 5500 rpm. The polymer used was copolymer of lactic and glycolic acid in ratio of 75% lactic acid and 25% glycolic acid, i. e., Resomer RG752H having inherent viscosity of 0.17 dl/g.

Table No. 15 Details on formulation trials conducted using single emulsion solvent evaporation method use of dimethyl formamide as drug solvent.

Sr.	Ingredients /Batch No.		mg/vial	EP/33	EP/34	EP/37	EP/40	EP/49*
Batch size			--	10 vials				34 vials
Drug: polymer ratio			--	1:8.8	1:4.6	1:2.3	1:4.6	1:4.6
1	**Drug solution**	Eptifibatide	12. 96	0.13 g	0.13 g	0.13 g	0.13 g	0.44 g
2		DMFA (ml)	--	1	1	1	1	3.7
3	**Polymer solution**	PLGA 75:25	30-114	1.44 g	0.60 g	0.30 g	0.60 g	2.04 g
		DCM (ml)	--	1.5	1	1	1	3
5	**Aqueous Phase**	PVA	--	0.45 g	0. 45 g	0.45 g	0.45 g	1.60 g
6		Purified water (ml)	--	180	180	180	180	600
7	**Bulking Agent**	D-Mannitol	7.04	0.07 g	0.07 g	0.07 g	0.07 g	0.24 g
8		Purified water (ml)	--	10	10	10	10	34

The analytical data for the above trials is mentioned in the table below.

Table No. 16 Analytical data for trials conducted using single emulsion solvent evaporation method using dimethyl formamide.

Sr. No.	Batch No.		EP/34	EP/40	EP/49
	Test		Results		
1	Appearance		WLP	WLP	WLP
2	Assay Total drug content		95.40%	96.21%	101.3%
3	Assay Free content		0.02%	0.05%	0.02%
4	Particle Size Distribution d 50		25.12 µm	21.22 µm	14.21 µm
	Particle Size Distribution d 90		47.22 µm	48.51 µm	45.45 µm
5	SEM		Refer to the below images.		
6	Residual Solvent.	DCM (NMT 600 ppm)	487	510	362
		DMFA (NMT 800 ppm)	578	498	458

WLP: White lyophilised powder

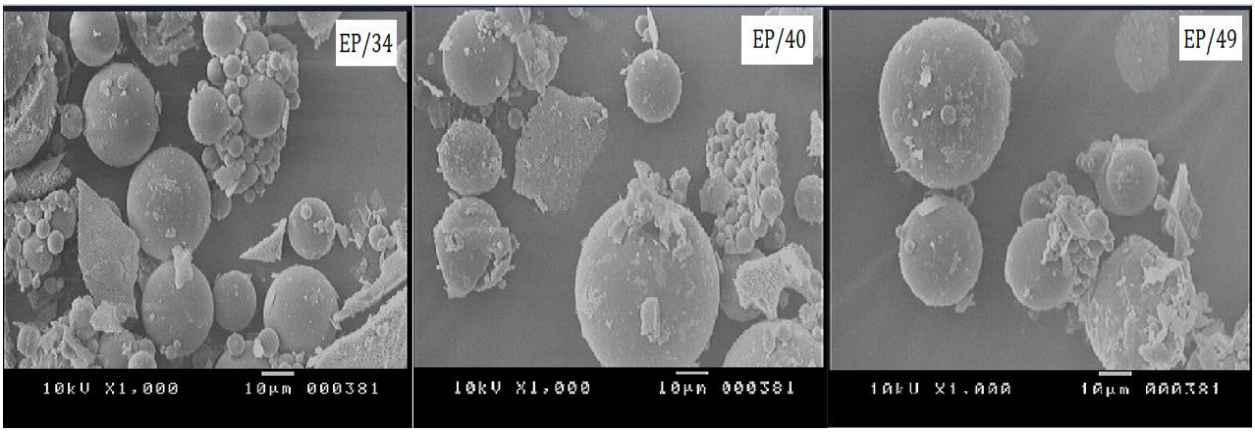


Figure No. 8 SEM images for Eptifibatide microspheres trials batches

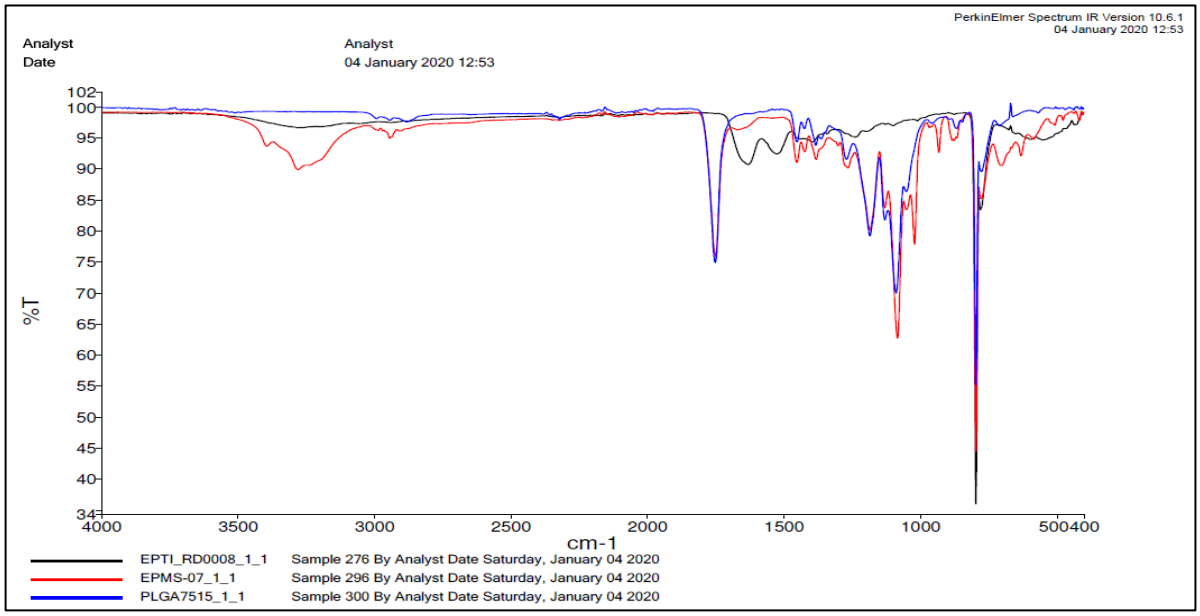
Biodegradable polymer is the key raw material of these delivery systems and is sold to pharma companies at high cost. Hence it is very much essential to first optimise the ideal amount of polymer which will give the desired drug entrapment. The first ratio tried 1:8.8 gave good drug entrapment of around 96%. The amount of polymer was reduced to get similar drug entrapment. Based on the above trials different drug-to-polymer ratios were evaluated and at all the ratios studied, good drug entrapment of about > 90% was seen. The free drug content of all the trials was very low which is desirable to avoid unwanted burst release. However, with the declining drug -to-polymer ratio as seen in trials no EP/37 marginal decline in drug load was seen. These being the initial trials and still other parameters like particle size and in vitro release were not yet evaluated to keep some working margin it was decided to use the drug-to-polymer ratio of 1:4.6 for future trials. One reproducible batch at higher scale was manufactured and evaluated.

- **Formulation trials using single emulsion solvent evaporation method-direct drug dispersion, solid dispersion and saturation of external phase.**

With the intent to keep the manufacturing process simple and to use less amount of organic solvent, direct dispersion of the drug in the polymer solution was evaluated. Also, solid dispersion of the drug and the polymer was studied. Here both the drug and the polymer were dissolved in dimethyl sulphoxide and the solvent was evaporated in turbo evaporator. The obtained solid dispersion was then directly dissolved in dichloromethane and then used in batch manufacturing. Saturation of the external PVA solution with some bulking agent, was also studied for better drug loading. The table below details the analytical data for trials.

Table No. 17 Analytical data for trials conducted using single emulsion solvent evaporation method by direct drug dispersion and saturation of external phase.

Sr. No.	Batch No.	Specification	EP/56	EP/57	EP/58	EP/59	EP/60	EP/62
	Batch Details		DD	SD*	EP/56 Repeat	DDS10	DDS5	DDS1
1	Total Drug content	90-110 % w/w	55.23	57.33	56.01	83.45	77.42	63.21
2	Free Drug content	NMT 1%	0.78	0.81	0.83	0.22	0.34	0.27
3	PSD d 50	NMT 25 µm	17.32	18.21	16.25	15.22	14.61	13.13
	PSD d 90	NMT 50 µm	45.31	48.19	48.25	43.56	44.89	45.92
4	DCM content	NMT 600 ppm	438	501	467	450	433	203
	DMFA content	NMT 800 ppm	340	389	401	340	430	389
	DMSO Content	NMT 5000 ppm	NA	299	NA	NA	NA	NA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EPMS-07_1_1 Sample 296 By Analyst Date Saturday, January 04 2020
PLGA7515_1_1 Sample 300 By Analyst Date Saturday, January 04 2020

Figure No. 9 IR graph for Eptifibatide Polymer solid dispersion

Eptifibatide is having slight solubility in water. For highly water-soluble drug direct dispersion of the drug into polymer solution will lead to poor entrapment due to complete partitioning of the drug into the external phase.[23] The same was observed in the above trials. Eptifibatide was directly added in the polymer solution to form a drug-polymer dispersion. In trial no. EP/56 very low drug entrapment was achieved clearly indicating the partitioning of the drug into the external aqueous phase thus causing poor drug entrapment. Hence, it was decided to first bind the drug to the polymer by using solid dispersion and other was to saturate the external aqueous phase with D-mannitol so the partitioning of the drug into the external phase is minimised and improves drug entrapment. Common solvent like dimethyl sulphoxide was used to dissolve both the drug and the polymer and the solvent was evaporated using turbo evaporator and dried to get a dried powder mass, which was then dissolved in DCM and then further used in the batch. The solid dispersion was evaluated for IR and solvent content before using in batch manufacturing. Saturating the external phase with D-mannitol did give a positive result with good entrapment being observed as the concentration of D-mannitol was increased in the external phase.(EP/59 with 10% Mannitol, EP/60 with 5 % mannitol and EP/62 with 1% Mannitol) This method can be used as an alternative to the existing method to improve the drug entrapment. However saturation at scale-up level can be a problem as very huge amount of D-mannitol will be required for saturation. Use of solid dispersion didn't help much.

- **Formulation trials using single emulsion solvent evaporation method -Use of polymer with high inherent viscosity (IV).**

To achieve desired drug entrapment (>85%w/w) polymer of higher inherent viscosity was used in the below trials. Polymers used for these trials include.

Resomer RG752H with an inherent viscosity of 0.17 dl/g
Resomer RG752H with an inherent viscosity of 0.20 dl/g
Resomer RG752H with an inherent viscosity of 0.25 dl/g

Table No. 18 Detail of trials conducted using polymer of higher Inherent viscosity manufactured using single emulsion solvent evaporation method.

Sr.	**Ingredients /Batch No.**		**mg/vial**	**EP/63**	**EP/64**	**EP/65**	**EP/66**
Batch Size			**--**	**10 vials**			
Polymer Inherent viscosity (dl/g)				**0.17**	**0.20**	**0.25**	**0.25**
1	**Drug solution**	Eptifibatide	12.96	0.13	0.13	0.13	0.13
2		DMFA (ml)	--	1	1*	1	1
3	**Polymer solution**	PLGA 75:25	60	0.60	0.60	0.60	0.60
4		DCM (ml)	--	1	1	1.5	1.5
5	**External Aqueous Phase**	PVA	--	0.45	0.45	0.45	0.45
6		Purified water (ml)	--	180	180	180	180
7		D-Mannitol	7.04	0.07	0.07	0.07	0.07

8	**Bulking agent**	Purified water	--	10 ml	10 ml	10 ml	10 ml

Table No. 19 Analytical data for trials conducted using polymers of higher IV

Sr. No.	Batch No. / Batch Details	Specification	EP/63 0.17 dl/g	EP/64 0.20dl/g	EP/65 0.25 dl/g	EP/66 0.25dl/g
1	Total Drug content	90-110 % w/w	85.66	89.45	91.33	93.12
2	Free Drug content	NMT 1%	0.01	0.03	0.04	0.04
3	PSD d 50	NMT 25 µm	15.07	16.21	17.34	15.34
	PSD d 90	NMT 50 µm	43.31	43.19	42.21	39.71

As was expected higher and better drug entrapment was achieved in trials no. EP/64 and EP/65. This was crosschecked by manufacturing a reproducible batch no. EP/66. Good entrapment was seen when polymer of higher inherent viscosity 0.25 dl/g was used instead of polymer of lower inherent viscosity of 0.17 dl/g. As stated earlier higher viscosity translates into high molecular weight which in turns translate into lower solubility. Thus when the dissolved polymer comes in contact with external aqueous phase rapid precipitation of the polymer occurs causing higher drug loading.

- **Formulation trials conducted using double emulsion method -use of 0.025 M Citrate and 0.01 M Hydrochloride buffer.**

To understand the impact of manufacturing method used these trials were conducted to check the impact of double emulsion solvent evaporation method. Here citrate buffer and hydrochloride buffers were used to dissolve the drug and then then use in the process.

Table No. 20 Details on trials conducted using double emulsion solvent evaporation. method.

Sr. No.	Ingredients /Batch No.		mg/vial	EP/84	EP/85	EP/86	EP/86R*
Batch Size			--	**10 vials**			
1	Drug Solution	Eptifibatide	12.96	0.13	0.13	0.13	0.13
2		0.025 M CB/ 0.01 M HCl* (ml)	--	1	1 *	2	2
3	Polymer Solution	PLGA 75:25	60	0.60	0.60	0.60	0.60
4		DCM (ml)	--	1	1	1	1
5	External aqueous phase	PVA	--	0.45	0.45	0.45	0.45
6		Purified water (ml)	--	180	180	180	180
7	Bulking agent	D-Mannitol	7.04	0.07	0.07	0.07	0.07
8		Purified water (ml)	--	10	10	10	10

Description for abbreviations used in the table: CB: Citrate buffer, HCl-Hydrochloride buffer.

Table No. 21 Analytical data for trials conducted using double emulsion method.

Sr. No.	Batch No. / Batch Details	Specification	EP/84	EP/85	EP/86	EP/86R
			Citrate	HCl	Citrate	Citrate
1	Total Drug content	90-110 % w/w	97.34	96.81	96.51	97.13
2	Free Drug content	NMT 1%	0.16	0.18	0.31	0.21
3	PSD d 50	NMT 25 µm	34.98	35.55	37.70	28.33
	PSD d 90	NMT 50 µm	55.66	56.23	55.30	44.34
4	Residual DCM	NMT 600 ppm	251	289	294	312
5	Residual DMFA	NMT 800 ppm	399	412	420	357
6	Residual Methanol	NMT 3000 ppm	ND	ND	ND	ND

Trial no. EP/84 is a batch formulated using citrate buffer as drug solvent, where it gave good drug entrapment. When compared to trial No. EP/85 which is formulated with 0. 01 N HCl buffer not much difference was seen in terms of drug entrapment. However, higher particle size was obtained in all these trials. High drug load was reconfirmed in trial no. EP/86 and EP/86R. The manufacturing method used to formulate microspheres has a profound impact on the overall physicochemical attributes of the microspheres formed. Microspheres formulated using single emulsion solvent evaporation method trial no. EP/58 were less porous when compared to microspheres formulated using double emulsion solvent evaporation method. Double emulsion solvent evaporation method gave good drug entrapment as partition of the drug into the external phase is prevented due to the presence of the organic phase which acts as barrier for the drug to pass to the external phase. Microspheres formulated with double emulsion solvent evaporation method gave higher particle size when compared to batches formulated with single emulsion method. This was due to high drug loading into these microspheres. The overall release profile of drug from these microspheres was faster probably due to high porosity. With double emulsion method, huge processing losses observed with this manufacturing method as mixing the aqueous drug solution into the organic polymer solution forms a milky white emulsion which is viscous and difficult to transfer. Thus, even though double emulsion solvent evaporation method gives good drug entrapment, the yield from the process is compromised. Since the polymer used is very expensive, such processing losses won't be advisable or approaches to reduce this processing losses must be used.

- **Formulation trials conducted using Coacervation phase separation method.**

In continuation to evaluate the impact of the manufacturing process and to have better drug loading following batches were formulated using the coacervation phase separation method

Table No. 22 Details of trials conducted using coacervation phase separation method

Sr.	Ingredients /Batch No.		mg/vial	03	14	18	19	21	22	23	24
Batch Size			--	10 vials							
Drug /solvent/Drug: Polymer ratio				Direct	DMSO	DMFA	DMFA	1:8	1:4	1:6	1:6
1	Drug Solution	Eptifibatide	12.96	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
2		DMFA/ DMSO* (ml)	--	---	2.2 l*	1	1	1	1	1	1
3	Polymer Solution	PLGA 75:25/504H#	114.04	1.14	1.14	1.14	1.14#	1.14	1.14	1.14	1.14
4		DCM (ml)	--	20	20	20	20	25	20	20	20
5	Anti solvent	Medical Fluid (1000 cst/ 500 cst)#	--	14.68	14.68	15.0	22.11#	14.68	14.68	14.68	14.68
6	Hardening Agt.	Miglyol 84 (ml)	--	100	100	100	100	100	100	100	100
7	Solvent	Iso propyl alcohol (ml)	7.04	200	200	200	200	100	200	200	200
8	Surfactant	Polysorbate 80 (0.1%w/v)	--	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table No. 23 Analytical data for trials conducted using coacervation phase separation. method.

Sr. No.	Ingredients		mg/vial	EP/03	EP/14	EP/18	EP/19	EP/21	EP/22	EP/23
	Drug: Polymer			Direct	DMSO	DMFA	DMFA	1:8	1:4	1:6
1	Total Drug content		90-110 % w/w	85.66	89.45	92.11	93.12	98.23	98.20	97.12
2	Free Drug content		NMT 1%	0.01%	0.02%	0.01%	0.01	0.01	0.01	0.00
3	PSD d 50		NMT 25 µm	67.34	71.30	74.13	68.12	60.12	54.20	63.22
	PSD d 90		NMT 50 µm	122.12	113.56	112.19	113.44	110.34	108.10	115.45
4	Residual Solvent (ppm)	DCM	NMT 600	890	877	756	834	750	867	749
		DFMA	NMT 800	1003	690	801	744	550	578	621
		Methanol	NMT 3000	NA	NA	NA	NA	ND	ND	ND

DMFA: Dimelthyl formamide, DMSO: Dimethyl sulfoxide Medical Fluid A: 1000 Cst B: 500 Cst

Coacervation phase separation process is another process where the processing parameters are very crucial and change in one process parameter will have great impact on the critical quality attributes. Two solvents were evaluated for the drug DMFA and DMSO and not much difference was seen when these solvents were used (EP/14 and EP/18). Medical fluid which basically acts as antisolvent for the polymer, two different viscosity grades 1000 Cst and 500 Cst were also evaluated (EP/18 & EP/19). High viscosity medical fluid causes immediate precipitation of the polymer when compared to that against low viscosity. The amount of

medical fluid required is less when higher viscosity grade is used. Overall good drug entrapment was achieved in all the formulated batches. Good drug entrapment was achieved even when the drug to polymer ratio was lowered to 1:4 (EP/22). The drug was also added directly to the polymer solution in trials no EP/03. The biggest advantage of using this process was negligible free drug, which is very much essential to avoid initial burst release of the drug. The microspheres formed were aggregated and having dimpled surface than those formed using solvent evaporation method. This is basically due to use of medical fluid which gives an oily texture, and this causes aggregation of the microspheres causing them to stick to each other and give the dimpled surface. The release profile obtained by microspheres formulated by this method is more controlled and gradual with no burst release. Phase separation evaporation method is a possible alternative to other methods. The only disadvantage for this method is requirement of large quantity of organic solvent and aggregation of the particles which makes the removal of residual solvent difficult. All the batches showed higher residual solvent content which can be addressed with longer solvent evaporation cycle and also by temperature exposure during drying/lyophilisation. Handling of large amount of organic solvent at scale up level is again troublesome. Environmental monitoring and disposing of this organic solvent safely is quite challenging. Aggregation of the microspheres gives higher residual solvent content which again might causes toxicity, and thus need more aggressive steps to control them within the specified ICH limits.[24]

All the batches formulated using different manufacturing methods were characterised and the compiled data is mentioned below.

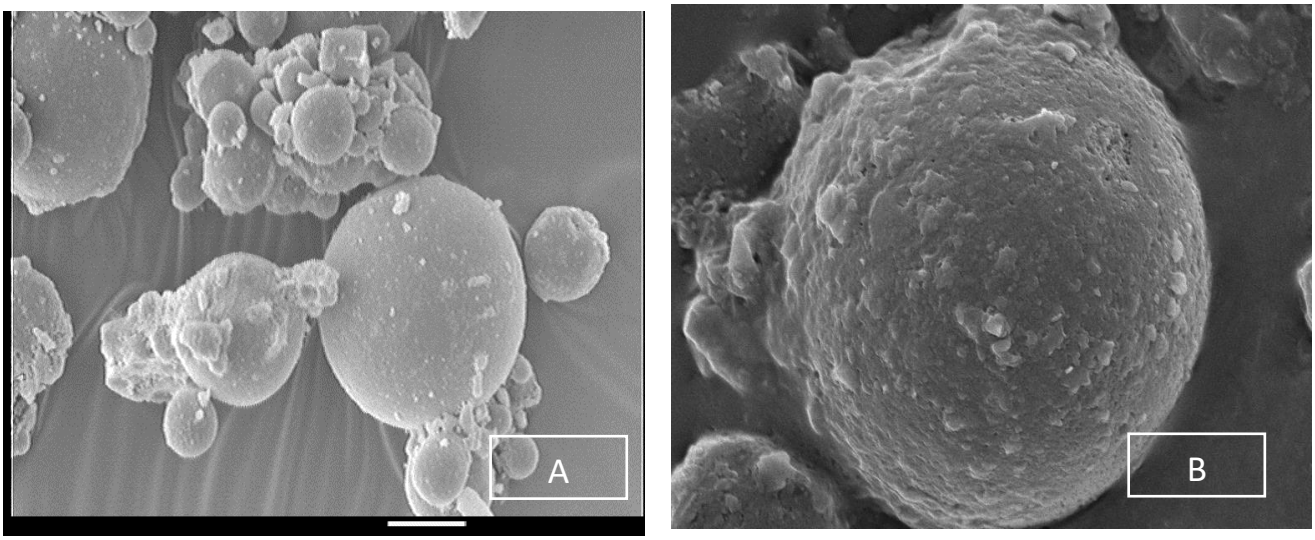

Figure No. 10 SEM images for microspheres formulated using single emulsion method (A) and double emulsion method (B)

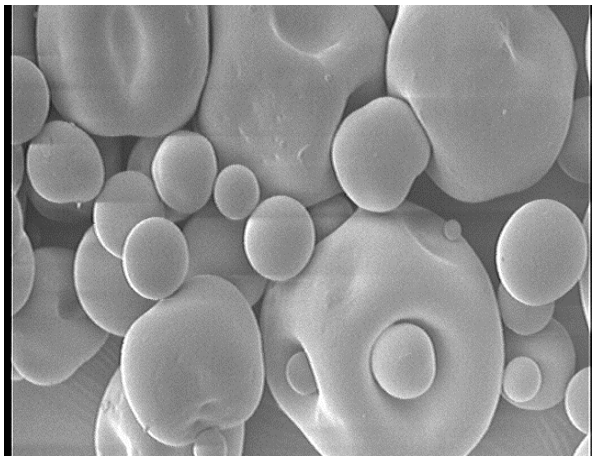

Figure No. 11 SEM images for microspheres formulated using coacervation phase separation method

- **Formulation trials using the inclusion of additives.**

To address the issues of drug entrapment, drug release and drug stability the impact of inclusion of additives in the formulation was evaluated. These additives were added either in the drug phase or the polymer phase.

Inclusion of additives in the drug phase

Three additives were chosen to be added in the drug phase namely Hydroxylproply-β-cyclodextrin (HPβCD), Trehalose, and Carboxy methyl cellulose sodium (Na-CMC). These additives were studied at three different ratios with respect to the amount of drug used. The amount of solvent used to dissolve both the drug and the additives was adjusted to prevent any precipitation of the dissolved components.

- HPβCD

The basic intention of using HPβCD was to have good drug loading and also to evaluate its impact on the burst release seen.

Table No. 24 Details of trials conducted with the inclusion of additives in the drug phase.

Sr	**Ingredients**	**mg/vial**	**78**	**79**	**80**	**81**	**88**	**89**	**105**	**87**	**103**	**104**
Batch Size			**10 vials**				**10 vials**			**10 vials**		
Additives			**HPβCD +Drug**				**Trehalose + Drug**			**Na-CMC+ Drug**		
Drug: Additive Ratio			**1:0.5**	**1:1**	**1:2**	**1:1 R**	**1:0.5**	**1:1**	**1:2**	**1:0.5**	**1:1**	**1:2**
1	Eptifibatide	12.96	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
2	**Additives**		0.07	0.13	0.26	0.13	0.07	0.13	0.26	0.07	0.13	0.26
3	DMFA (ml)	--	1	1.5	2	1.5	1	1.5	2	1	1.5	2.5
4	PLGA 75:25	60	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
5	DCM (ml)	--	1	1	1	1	1	1	1	1	1	1.5
6	PVA	--	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
7	Water (ml)	--	180	180	180	180	180	180	180	180	180	180
8	D-Mannitol	7.04	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
9	Water (ml)	--	10	10	10	10	10	10	10	10	10	10

Table No. 25 Analytical data for trials conducted with the inclusion of Hydroxy propyl beta cyclodextrins.

Sr. No.	Batch No. / Drug: HPBCD	Specification	EP/78	EP/79	EP/80	EP/81
			1:0.5	1:1	1:2	1:1
1	Total Drug content	90-110 % w/w	95.2	97.6	96.1	98.2
2	Free Drug content	NMT 1%	0.01	0.02	0.01	0.02
3	PSD d 50	NMT 25 µm	14.15	18.34	19.81	18.11
	PSD d 90	NMT 50 µm	46.87	48.30	43.46	47.23
4	In vitro release in Lactic Acid buffer pH 2.2	8 hours	24.21	15.13	10.23	16.22
		12 hours	27.18	31.10	30.15	33.45
		24 hours	64.12	56.14	55.34	59.34
		48 hours	94.47	90.21	91.35	88.22

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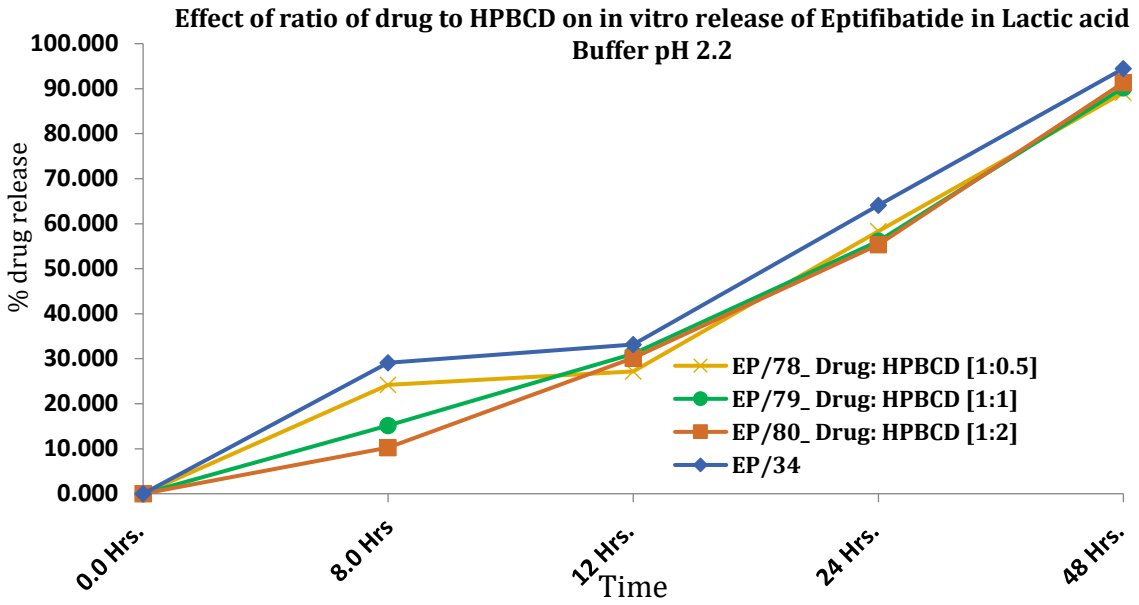

Figure No. 12 Comparative in vitro release profile of Eptifibatide microspheres with different ratios of drug to HPβCD

Complexing the drug with HPβCD gave good entrapment (>85%) at all the ratios studied. Presence of HPβCD gave very low free drug content. HPβCD not only helped in giving good drug entrapment but also it controlled the unwanted initial burst release. Presence of HPβCD in the formulation gave a more gradual release when compared to the batch without HPβCD. The possible reason for this gradual release is due to the complex/bonding formed between the drug and HPβCD. Thus, inclusion of HPβCD not only helped to improvise the drug entrapment but it also played a good role in achieving gradual release of the drug from the microspheres.

Table No. 26 Comparative in vitro release profile of conventional Microspheres vs microspheres formulated with inclusion of HP-β-CD.

Batch No.	EP/81	EP/34	EP/55
Batch Details	Microspheres with HP-β-CD	Microspheres with out additives	Microspheres with mPEG
15 mins	2.31	3.12	13.45
30 mins	2.93	4.55	14.67
1 Hrs.	4.33	7.43	16.13
8 Hrs	10.41	34.67	30.45
12 Hrs	31.33	38.60	33.46
24 Hrs.	55.45	58.10	65.82
48 Hrs.	91.34	74.30	84.15

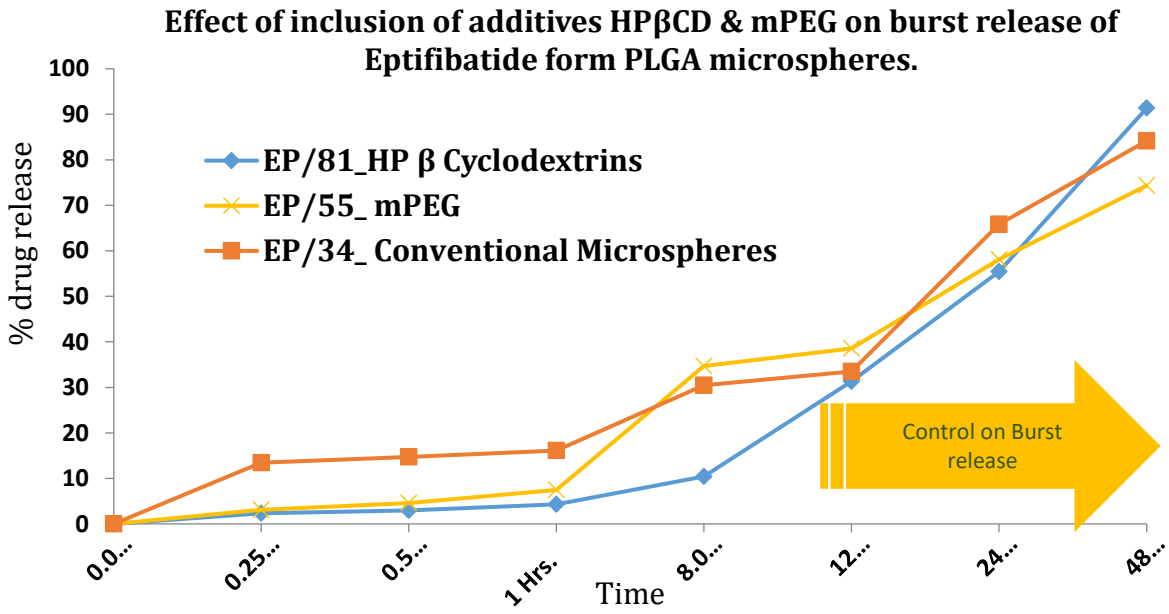

Figure No. 13 Comparative in vitro release profile of Eptifibatide microspheres effect of inclusion of additives on burst release

Inclusion of HPβCD in the drug phase has helped control the initial burst as is seen in the graph. This is basically due to binding of the drug by the HPβCD.

- **TREHALOSE**

The basic intension of adding Trehalose was to increase the stability of the drug inside the microspheres during storage and during manufacturing process.

Table 27 Analytical data for trials conducted to evaluate the effect of combining drug with Trehalose.

Sr. No.	Batch No.	Specification	EP/88	EP/89	EP/105
	Drug: Trehalose		1:0.5	1:1	1:2
1	Total Drug content	90-110 % w/w	89.1	93.2	90.5
2	Free Drug content	NMT 1%	0.01	0.02	0.01
3	PSD d 50	NMT 25 µm	14.28	19.20	18.21
	PSD d 90	NMT 50 µm	47.32	48.67	46.12

Trehalose is known as stabiliser. Its presence in the formulation did not affect much of the drug loading. The main intention of using trehalose in the formulation was to see its effect on drug stability. Eptifibatide is a peptide, and it is known to undergo structural changes due to use of organic solvent and harsh manufacturing conditions employed. Biodegradable polymers undergo degradation to form lactic acid and water thus lowering the pH inside the microspheres. This is one of the reasons why drug which undergoes degradation in acidic condition need to have some kind of neutralizing agent to be added in the formulation. Thus, complexing the drug with Trehalose can be one of the approaches to improve drug stability. As such no big difference was seen in terms of release profile when compared to the batch formulated without using Trehalose.

- **CARBOXY METHYL CELLULOSE SODIUM**

This third additive was evaluated as it had viscosity increasing properties so inclusion in the formulation will prevent the partition of the drug in the external phase.

Table 28 Analytical data for trials conducted to evaluate the effect of inclusion of carboxy methyl cellulose sodium

Sr. No.	Batch No.	Specification	EP/87	EP/103	EP/104
	Drug: Sodium CMC		1:0.5	1:1	1:2
1	Total Drug content	90-110 % w/w	95.3	94.8	95.1
2	Free Drug content	NMT 1%	0.01	0.01	0.02
3	PSD d 50	NMT 25 µm	17.25	16.14	15.21
	PSD d 90	NMT 50 µm	43.21	45.18	45.11

Table No. 29 Comparative in vitro profile showing control of burst release with inclusion of HP-β-CD and carboxy methyl cellulose sodium.

Batch No.	EP/81	EP/34	EP/103
Batch Details	**Microspheres with HP-β-CD**	**Microspheres without additives**	**Microspheres with NaCMC**
15 mins	2.31	13.45	3.22
30 mins	2.93	14.67	7.55
1 Hrs.	4.33	16.13	15.40
8 Hrs	10.41	30.45	20.22
12 Hrs	31.33	33.46	31.00
24 Hrs.	55.45	65.82	61.04
48 Hrs.	91.34	84.15	87.40

Here also three different ratios were tried, inclusion of sodium carboxymethyl cellulose did give good entrapment however not much effect was seen on the release profile. Sodium carboxymethyl cellulose is viscosity increasing agent, hence its inclusion in the drug solution will cause an increase in viscosity and will lower the partitioning of the drug to the external aqueous phase thus improving the drug entrapment. Inclusion of sodium carboxymethyl cellulose also helped to control the initial burst seen at early time proving its need as an additive. Sodium carboxymethyl cellulose is known to have wound healing action, it inclusion in the formulation will also improvise the delivery of the drug through needles. Thus, inclusion of sodium carboxymethyl cellulose will give double benefit.

Inclusion of additives in the polymer phase.

Three additives were chosen to be added to the polymer phase, namely methoxy polyethyleneglycol (mPEG), Polyvinyl alcohol (PVA) and Polyethylene glycol (PEG 400) These additives were also studied at three different ratios with respect to the amount of polymer used. The amount of solvent used to dissolve both the polymer and the additives was adjusted to prevent any precipitation of the dissolved components and to keep the viscosity of the solution low/manageable.

Table No. 30 Details of trials conducted with inclusion of additives in the polymer phase.

Sr.	Ingredients	mg/vial	54	55	108	109	90	106	107	110	111	112
Batch Size			**10 vials**				**10 vials**			**10 vials**		
Additives			**mPEG + Polymer**				**PVA + Polymer**			**PEG 400 +Polymer**		
Polymer: Additive Ratio			**1:0.5**	**1:0.5**	**1:1**	**1:2**	**1:0.5**	**1:1**	**1:2**	**1:0.5**	**1:1**	**1:2**
1	Eptifibatide	12.96	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
2	DMFA (ml)	--	1	1	1	1	1	1	1	1	1	1
3	PLGA 75:25	60	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
4	**Additives**		0.3	0.3	0.6	1.2	0.3	0.6	1.2	0.3	0.6	1.2
5	DCM (ml)	--	2	2	2	3	2	2	2.5	2	2	2.5
6	PVA	--	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
7	Water (ml)	--	180	180	180	180	180	180	180	180	180	180
8	D-Mannitol	7.04	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
9	Water (ml)		10	10	10	10	10	10	10	10	10	1

- **methoxy PEG phospholipid.**

With an intention to study the impact of the inclusion of mPEG phospholipid on the drug entrapment, release, and other physicochemical parameters, formulations with and without mPEG phospholipid were made and compared. The basic intention of using mPEG was to see its effect in the lag phase of the polymer.

Table No. 31 Analytical data for trials conducted with inclusion of methoxy PEG phospholipid.

Sr. No.	Batch No.	Specification	EP/54	EP/55	EP/108	EP/109
	Drug: mPEG		1:0.5	1:0.5	1:1	1:2
1	Total Drug content	90-110 % w/w	94.23	95.1	93.44	92.31
2	Free Drug content	NMT 1%	0.01	0.02	0.01	0.02
3	PSD d 50	NMT 25 µm	12.34	13.45	14.33	13. 22
	PSD d 90	NMT 50 µm	43.12	44.56	45.18	44.45

The results obtained were promising. Inclusion of the mPEG phospholipid gave a positive result by controlling the burst release and the release at the latter time points was improved

- **Polyvinyl alcohol**

PVA is a surfactant so its inclusion might have an impact on the lag phase of the polymer.

Table No. 32 Analytical data for trials conducted with inclusion of PVA

Sr. No.	Batch No.	Specification	EP/90	EP/106	EP/107
	Drug: PVA		1:0.5	1:1	1:2
1	Total Drug content	90-110 % w/w	95.2	93.23	92.13
2	Free Drug content	NMT 1%	0.01	0.02	0.01
3	PSD d 50	NMT 25 µm	12.10	13.51	14.12
	PSD d 90	NMT 50 µm	43.12	44.17	45.12

Polyvinyl alcohol is used as surfactant in solvent evaporation method. Complexing this surfactant with biodegradable polymer affects it wettability and have an impact on initial lag in

the release profile which is very commonly seen with microspheres. Presence of PVA indeed proved to reduce the lag phase by providing more wetting of the polymer.

- **PEG-400**

PEG 400 is a surfactant so its inclusion might have an impact on the lag phase of the polymer.

Table No. 33 Analytical data for trials conducted with inclusion of PEG- 400

Sr. No.	Batch No.	Specification	EP/110	EP/108	EP/109	EP/111
	Drug: PEG 400		1:0.5	1:1	1:2	1:1
1	Total Drug content	90-110 % w/w	94.4	93.24	93.22	94.56
2	Free Drug content	NMT 1%	0.01	0.02	0.01	0.02
3	PSD d 50	NMT 25 µm	14.21	15.12	15.14	16.01
	PSD d 90	NMT 50 µm	45.11	47.11	46.21	44.23

Polyethylene glycol 400 (PEG-400) is a surfactant. Complexing this surfactant with biodegradable polymer has similar effect was observd with mPEG.

Table No. 34 Comparative in vitro release profile of conventional microspheres vs microspheres formulated with inclusion of PVA and PEG-400

Batch No.	EP/90	EP/34	EP/111
Batch Details	Microspheres with PVA	Microspheres without additives	Microspheres with PEG-400
15 mins	15. 41	13. 45	17.52
30 mins	17. 12	14.67	18.45
1 Hrs.	20. 13	16.13	23.23
8 Hrs	45. 34	30.45	47.45
12 Hrs	55.43	33.46	58.34
24 Hrs.	68. 45	65.82	70.12
48 Hrs.	89. 35	84.15	88.86

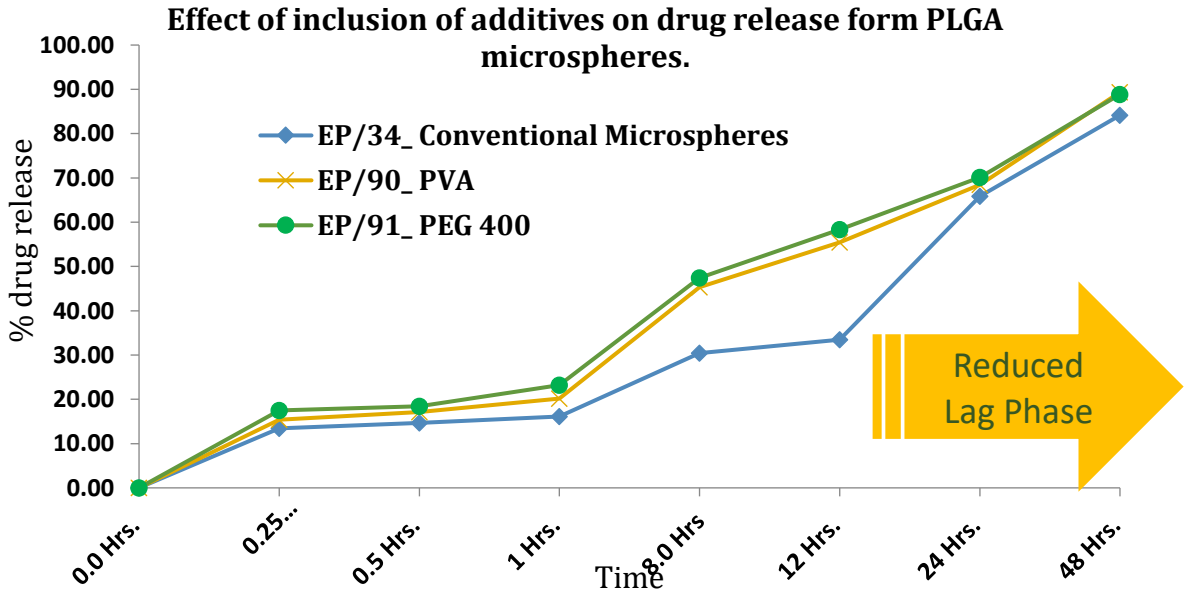

Figure No. 14 Comparative in vitro release profile of Eptifibatide microspheres effect of inclusion of additives on lag phase

Inclusion of PVA and PEG 400, both being plasticizer helped in wetting the polymer, thus the lag phase which is usually observed with these polymers is reduced and can be more beneficial.

- **Formulation trials conducted using polymer combinations/blending.**

Here two different polymers Resomer RG 503H and Resomer R202H were blended in different ratios to get polymers of different comonomer ratio. These blended polymers were than used to formulate microspheres. A single emulsion solvent evaporation method was used to formulate these batches. The blended polymer was evaluated for NR, inherent viscosity, and molecular weights.

One of many challenges with Biodegradable polymer based microspheres is the availability of the right grade of polymer to match the desired product specification. So, identifying the right grade and procuring this grade on a continuous basis for commercial supply is troublesome. Thus, there comes a need to use a combination of polymers to formulate a product that will give the desired tailor-made release.Three polymers were used by combining them in different ratios.

Resomer R202 H (100% DL- Lactic Acid) Inherent viscosity: 0.21 dl/g
Resomer RG502H (50 % DL-Lactide & 50 % Glycolide) Inherent viscosity: 0.21 dl/g
Resomer RG752H (75 % DL-Lactide & 25% Glycolide) Inherent viscosity: 0.17 dl/g
The polymers which had closer IV values were used. Following combinations were evaluated.

Table No. 35 Details of Polymer combination

Sr No.	Resomer R202H	Resomer RG502H	Amt of Lactic Acid	Amt of Glycolic Acid
1	100	0	100	0
2	90	10	95	5
3	80	20	90	10
4	70	30	85	15
5	60	40	80	20
6	50	50	75	25
7	40	60	70	30
8	30	70	65	35
9	20	80	60	40
10	10	90	55	45
11	0	100	50	50

The above blended polymers were evaluated for NMR, inherent viscosity, and molecular weight determination before using them in formulating lab batches.

Physicochemical Characterization of blended polymers.

Table No. 36 Physicochemical Characterisation of the blended Polymers.

Sr No.	Resomer R202H	Resomer RG502H	Amt of LA	Amt of GA	Appearance of solution	Inherent viscosity (dl/g)	Molecular weight (Daltons)
1	100	0	100	0	Clear Viscous	0.20	18000
2	90	10	95	5	Clear Viscous	0.20	19240
3	80	20	90	10	Clear Viscous	0.19	18500
4	70	30	85	15	Clear Viscous	0.18	17600
5	60	40	80	20	Clear Viscous	0.19	16300
6	50	50	75	25	Clear Viscous	0.18	15100
7	40	60	70	30	Clear Viscous	0.20	16780
8	30	70	65	35	Clear Viscous	0.21	22010
9	20	80	60	40	Clear Viscous	0.22	24024
10	10	90	55	45	Clear Viscous	0.25	23001
11	0	100	50	50	Clear Viscous	0.32	25000

Form the analytical data it was clear that when mixed in proper ratios polymer of desired viscosity and molecular weight can be achieved. The blended polymers were found to give desirable parameters matching closely in inherent viscosity and molecular weight.

Table No. 37 Details of trials conducted with blended polymers.

Sr.	**Ingredients /Batch No.**		**mg/ vial**	**74-1**	**74-3**	**74-5**	**74-6**	**74-7**	**74-9**	**74-11**	**74-12**
Comonomer ratio (LA:GA)				**100:00**	**95:5**	**80:20**	**75:25**	**70:30**	**60:40**	**50:50**	**75:25**
Polymer Blend (502H:202H)				**0:100**	**10:90**	**40:60**	**50:50**	**60:40**	**80:20**	**100:00**	**As such**
Batch Size			--	**10 vials**							
1	Drug	Eptifibatide	12.96	0.13	0.13	0.13	0.13	0. 13	0. 13	0.13	0.13
2	Solution	DMFA (ml)	--	1	1	1	1	1	1	1	1
3	Polymer	PLGA 75:25	60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
4	Solution	DCM (ml)	--	2	2	1.5	1.5	1.5	1.5	1	1.5
5	External	PVA	--	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
6	Aq. Phase	Water (ml)	--	180	180	180	180	180	180	180	180
7	Bulking	D-Mannitol	7.04	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
8	agent	Water (ml)	--	10	10	10	10	10	10	10	10

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Table No. 38 Analytical data for trials conducted using blended polymers (EP/74)

Sr.	**Ingredients**	01	02	03	04	05	06	07	08	09	10	11
Combination		1	2	3	4	5	6	7	8	9	10	11
1	Polymer R502H	0	10	20	30	40	50	60	70	80	90	100
	Polymer R202H	100	90	80	70	60	50	40	30	20	10	0
2	Total drug %	95.1	94.5	96.5	97.3	92.4	95.1	92.3	94.1	96.5	92.5	94.3
3	Free drug %	0.23	0.21	0.20	0.21	0.21	0.15	0.19	0.16	0.23	0.33	0.31
4	PSD D 50 µ	22.4	25.5	26.5	22.6	21.4	23.7	21.9	22.0	21.4	25.5	24.3
	PSD D 90 µ	43.5	44.7	43.7	43.5	43.6	48.6	46.2	44.8	45.6	44.1	47.2

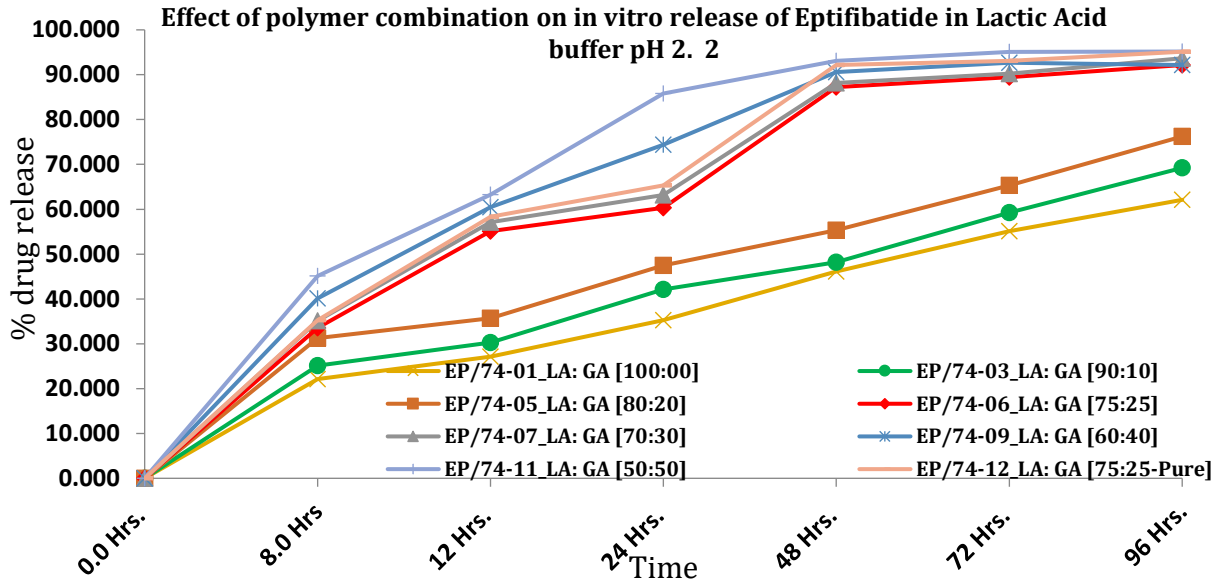

Figure No. 15 Comparative in vitro release profile of Eptifibatide microspheres Effect of polymer combinations

All the polymer combinations gave the same comonomer ratio as was expected while blending the polymer. There was exact corelation and overlay for Polymer combination 6 against the pure polymer Resomer RG 752H, which has Poly (dl- Lactic acid) and poly glycolic acid in ratio of 75:25. Thus physical blending of the polymer gave us the same result as was seen with pure polymer. IV and molecular weight plays a crucial role in drug release, same IV polymer with different comonomer ratio gives different release profile. R202H and RG502H polymers can be combined to get different ratios of polymer and in turn the release can be tailored.

Comparative physicochemical characterization of above developed formulations.

The above batches were evaluated the details of the same are mentioned in the table below**.**

Table No. 39 Complete physicochemical characterisation of developed batches.

Sr. No.	Batch No.	EP/49	EP/59	EP/66	EP/86	EP/23	EP/81	EP/55	EP/103	EP/90	EP/91
		SE	DDS	HIV	DE	CAPS	HBCD	MPEG	CMC	PEG	PVA
1	Total Drug	101.3	83.45	93.12	97.13	97.12	98.2	95.1	94.8	95.2	92.3
2	Free Drug	0.02	0.02	0.04	0.21	0.00	0.02	0.02	0.01	0.01	0.01
3	PSD d50	14.21	15.22	15.34	28.33	63.22	18.1	13.45	16.14	12.10	13.2
	PSD d90	45.45	43.56	39.71	44.34	115.45	47.23	44.56	45.18	43.12	44.11
4	DCM	362	450	413	312	749	324	289	301	256	301
5	DMFA	458	340	405	--	621	436	423	450	452	302
6	15 min	10.24	--	--	21.44	--	2.31	13.45	3.22	15.42	17.52
	30 min	13.22	--	--	22.34	--	2.93	14.67	7.55	17.12	18.45
	1 hr	19.40	--	--	31.00	--	4.33	16.13	15.40	20.13	23.23
	8 hr	25.16	22.41	16.72	39.54	10.97	10.41	30.45	20.22	45.34	47.45
	12 hr	35.58	36.78	30.14	38.56	28.63	31.3	33.46	31.33	55.43	58.34
	24 hr	67.62	69.13	45.19	68.75	43.6	55.45	65.82	61.04	68.45	70.12
	48 hr	87.85	91.13	78.45	94.81	81.05	91.34	84.15	87.40	89.35	88.86
7	TG	42.6	43.1	41.6	43.3	44.1	41.2	43.1	41.4	40.4	41.4
8	Mol Wt.	14563	13245	14623	12450	13104	1423	14350	13560	12560	13897
9	Moisture	1.45%	1.60%	1.56%	1.81%	1.15%	1.72%	1.45%	1.23%	1.11%	1.34%
10	SEM	Yes	--	--	Yes	Yes	Yes	Yes	Yes	Ye s	Yes
11	Total Imp	1.12	1.35%	1.31%	1.13%	2.03%	1.96%	1.78%	1.63%	1.32%	1.11%

SE-Single Emulsion, DE-Double emulsion, DDS-Direct drug & Saturation, HIV: High IV polymer CAPS-Coacervation phase separation.

The single emulsion solvent evaporation method is the most widely used and easy to scale up method. Thus, initial batches were formulated using the same method. The phase separation coacervation method is the second most widely known method, this method was also used during development. The presence of the drug in solubilized form is the key thing in achieving good drug entrapment. Thus, the selection of solvent for the drug was the critical and first step in development. The lab trials involved optimizing the drug to polymer ratio, the drug solution to polymer solution ratio, and the internal-to-external phase ratio to get the desired physiochemical parameters.

Optimization of PLGA Based Microspheres using Design of Experiments (DOE)

The developed batch of Eptifibatide microspheres was optimised by considering three independent variables which were,

- Ratio of drug to polymer,
- Ratio of internal to external phase, and
- Homogenisation speed

The impact of these variables was on evaluated on drug entrapment, particle size using Contour plots were plotted using statistical software Design of Experiment.

Figure No. 16 Contour plots for formula optimisation

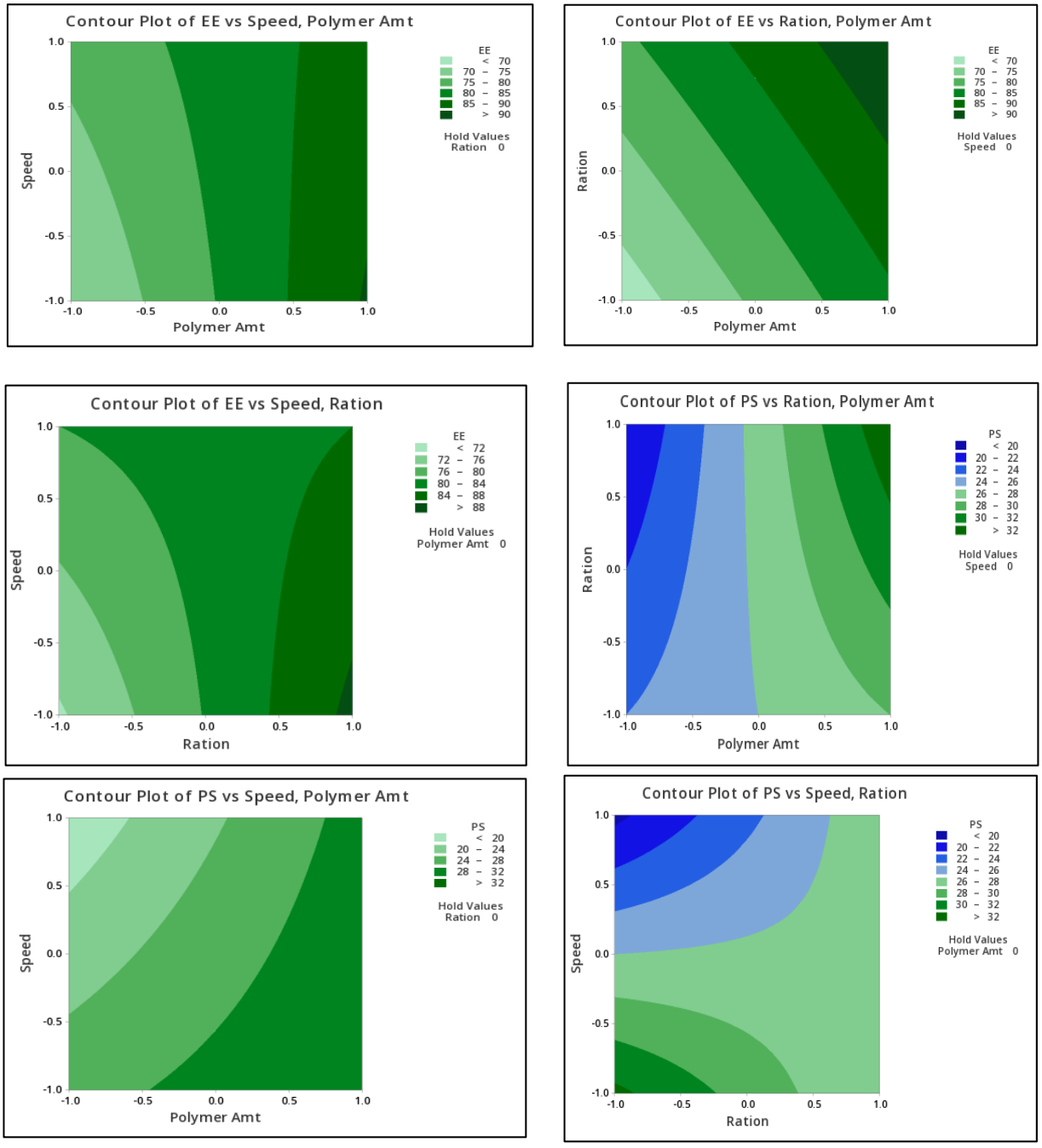

Table No. 40 Analysis of variance (ANOVA) test for each experimental response

Responses	Sum of squares	df	Mean squares	F value	P value	R^2	Adjusted R^2
Y1	0.024	3	7.743	6.43	0.0042	0.9633	0.9323
Y2	0.073	3	0.028	7.96	0.0365	0.9425	0.8938

Table No. 41 Optimised formulation condition offered by software.

				Predicted values		Experimental values	
Batch No.	X1	X2	X3	PS	EE	PS	EE
1	60 mg	1:90	500	23.5µ	95.12	22.5 µ	96.14

Table no. 42 Coded and actual values of variables used in CCD

Independent variables	Levels		
	-1	0	1
(A)Drug to Polymer Ratio	50 mg	60 mg	70 mg
(B) Ratio internal: external Phase	1:80	1:90	1:100
(C) Homogenisation speed	400	500	600
Dependent variables		Units	Constraints
Y1 Particles Size	PS	µm	Minimise
Y2 Entrapment Efficiency	EE	%w/w	Maximise

Table no. 43 Characterization parameters for Eptifibatide microspheres

Trails details	PS (µ± S. D.)	EE (%± S. D.)	Release
PLGA Msp	22.5 µ	96.14	NLT 85%

ANOVA results show model is significant, with predicted R^2 of 0.8076 being in reasonable agreement with adjusted R^2 of 0.8824. Drug to polymer ratio and homogenization speed and their interaction are significant ($p < 0.05$). High amount of drug decreases loading as the quantity of the polymer is not sufficient to entrap the entire amount. High ratio of external phase increased entrapment due to rapid extraction of organic solvent causing rapid precipitation of the polymer and thus increased loading. Higher homogenization speed reduced the entrapment and decreased the particles size.

Composition of optimised Eptifibatide microspheres.

The based on the development work and followed by optimization of the batches using design of experiment following composition was finalised.

Table 44 Composition for Optimised Eptifibatide Microspheres.

Sr. No.	Ingredients	Role	mg/vial	%w/w
1	Eptifibatide	Active Pharmaceutical Ingredients	12. 96	16. 2
2	Dimethyl formamide	Solvent for drug.	--	--
3	PLGA 75:25 Polymer	Biodegradable Polymer	60	75
4	Dichloromethane	Solvent for Polymer.	--	--
5	D-Mannitol	Bulking agent/Osmotic agent	7.04	8.8
Total weight of Lyophilized powder per vial			80 g	

The optimised batch of microspheres was charged on Stability studies and was found to be stable.

8. FORMULATION OF EPTIFIBATIDE IMPLANTS

As defined in the objectives one phase of this research projects involved development of biodegradable polymer-based Implant. To decide the required amount of polymer needed for this, few trials were planned with different ratio of drug to polymer. To begin with here also the ratio of drug to polymer was titrated to achieve desirable drug loading and also the desired physical form and finally the desired release profile.

Table No. 45 Details of trials conducted to formulate Eptifibatide implants.

Sr. No.	**Batch No.**	**----**	**EP/51**	**EP/52**	**EP/53**
	Batch Size	**----**	**80 Implants**		
	Drug: polymer Ratio	**----**	**1:1**	**1:2**	**1:3**
	Extrusion Temperature		**80° C**		
	Extrusion Speed		**60-70 rpm (5-7 N/m^2)**		
	Ingredients	**Role**	**mg/ implant**		
1	Drug	Active	12.96	12.96	12.96
2	Resomer RG 752H	Polymer	13.04	26.04	39.04
3	Total weight of Implant		26	39	52

The analytical data for extruded implants is mentioned in the table below.

Table No. 46 Analytical data for Eptifibatide implant formulated using. different drug to polymer ratios.

Sr. No.			**Implants Trials**		
	Batch No.		**EP/51 (1:1)**	**EP/52 (1:2)**	**EP/53 (1:3)**
	Drug to Polymer Ratio		1:1	1:2	1:3
1	**Assay**		102.1%	99.35%	98.12%
2	**Weight of Implant**		26	39	52
3	**Diameter of implant (mm)**		1.1-1.2	1.2-1.3	1.1-1.2
	Length of implant (mm)		18	26	28
4	**In vitro release in lactic acid buffer pH 2.2**	0.0 Hrs.	0.00	0.00	0.00
		1.0 Hrs	6.44	5.43	2.33
		4.0 Hrs	10.32	8.34	5.64
		8.0 Hrs	15.32	13.45	10. 32
		12.0 Hrs	21.78	17.56	15.43
		24.0 Hrs	31.44	26.43	22.45
		33.0 Hrs.	45.78	38.66	31.23
		48.0 Hrs.	68.66	62.54	55.32
		60.0 Hrs.	82.18	75.89	71.43
		72.0Hrs.	87.67	80.75	77.34

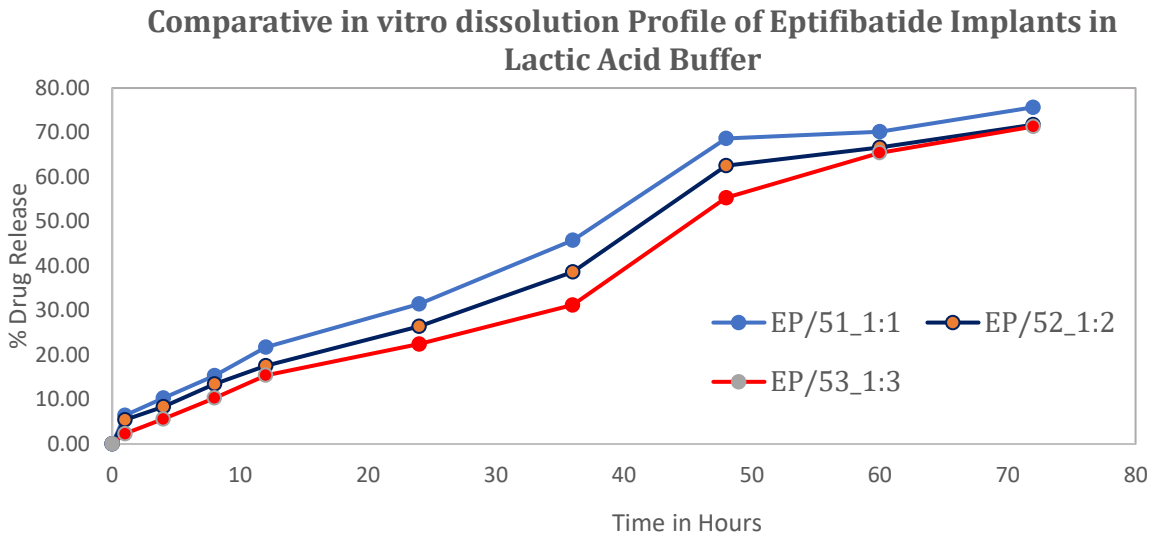

Figure No. 17 Comparative in vitro release profile of Eptifibatide implant in lactic acid buffer

All the three batches gave the desired drug content of more than 90 %. The in vitro release was more than 70% in 48 hours. As the ratio of drug to polymer increases the rate of dissolution decreased. In continuation of the above trials further optimisation was done in terms of processing, drug polymer mixture and the temperature of extrusion.

Trials to evaluate the impact of Extrusion temperature and physical form of the drug on the properties of Extruded implants.

The trials below evaluated the impact of the temperature during extrusion and also the impact of physical form of drug on the properties of implant.

Table No. 47 Details on trials conducted to evaluate the impact of extrusion temperature. and process on the Implants.

Batch No.	**EP/67**	**EP/68**	**EP/69**	**EP/70**	**EP/71**	**EP/72**
Drug: Polymer Ratio	1:3.01	1:3.01	1:3.01	1:3.01	1:3.01	1:3.01
Drug mixing with polymer	PM	PM	PM	PM	PM	LP
Temperature	82° C	90° C	70° C	90 °C	90° C	90° C
Ingredients	**mg/ imp**	**mg/ imp**	**mg/ imp**	**mg/ imp**	**mg/ imp**	**mg/ imp**
Drug	12.96	12.96	12.96	12.96	12.96	12.96
PLGA 75:25	39.04	39.04	39.04	39.04	39.04	39.04
Weight of Implant	52	52	52	52	52	52
Extrusion Parameters						
Temperature (°C)	82	90	70	82	82	82
Speed (rpm)	60-70	60-70	60-70	60-70	60-70	60-70
Torque (N/m^2)	5-7	5-7	5-7	5-7	5-7	5-7
Implant Dimensions (mm)	1.1-1.2	1.1-1.2	1.1-1.2	1.1-1.2	1.1-1.2	1.1-1.2

PM: Physical mixture & LP: Lyophilised form.

The analytical data of above trials is mentioned in the table below.

Table No. 48 Analytical data for trials conducted to study the effect of temperature. extrusion and processing on implants

Batch No.		EP/67	EP/68	EP/69	EP/70	EP/71	EP/72
Dimensions (mm)		1.1-1.2	1.1-1.2	1.1-1.2	1.1-1.2	1.1-1.2	1.1-1.2
Appearance of Implant		Off white	Off white	Transparent	Off white	Off white	Off white
Total Drug		101.1%	99.98%	99.23%	98.72%	98.21%	99.12%
In vitro release	1.0 Hrs	10.23	12.78	13.40	43.10	37.54	12.23
	8.0 Hrs	17.34	15.42	22.45	55.56	58.45	25.45
	12.0 Hrs	25.68	35.34	34.15	68.34	71.10	55.45
	24.0 Hrs	35.64	41.45	40.16	78.34	81.34	74.51
	48 Hrs.	71.06	77.45	76.15	87.56	94.34	89.45
	72 Hrs.	89.23	90.13	89.23	88.11	93.45	88.12

Extrusion at lower temperature gave transparent implant. This might probably be due to non-uniform blending of the drug and the melted polymer. Not much difference was observed on drug entrapment and release even when extruded at higher temperature, however when the implant blend was lyophilised together it gave incomplete release at latter time points. This might be due to uniform blending and binding of the drug and the polymer during when dissolved in common solvent and then lyophilised.

Composition of optimised Eptifibatide Implant

Based on the trials conducted and based on optimisation studies quantitative and qualitative composition of Eptifibatide implant was finalized.

Table 49 Composition of optimised Eptifibatide implant

Sr. No.	Ingredients	Role	mg/vial	%w/w
1	Eptifibatide	Drug	12.96	24.92
2	PLGA 75:25 Polymer	Biodegradable polymer	39.04	75.07
Total weight of Implant			52 mg	

A reproducible batch manufactured (B.No. EP/72) was charged on stability and was found to be stable.

9. FORMULATION OF EPTIFIBATIDE IN SITU IMPLANT

To fulfil one of the objectives of development of in situ implant of Eptifibatide following activities were conducted. Formulation of in situ implant can be done in two ways, either the drug can be dissolved in the polymer of it can be kept separated and mixed with the dissolved polymer before injection. The stability of drug dissolved/ dispersed is polymer solution was evaluated.

Table No. 50 Stability of Eptifibatide with polymer in NMP

Batch No. EP/91	Time	Drug Stability in NMP Solvent	
	Temperature	2-8 C	Room temperature
Assay	Initial	97.40%	97.40%
	Day 4	97.20%	96.21%
	Day 10	97.11%	95.71%
	Day 15	96.44%	94.32%
	Day 20	95.12%	90.23%
	Day 30	94.19%	87.45%
	% Drop in assay	3.21%	9.95%

Eptifibatide when dispersed with polymer in NMP undergo degradation both at 2-8 C and at room temperature. Thus, to formulate Eptifibatide in situ implant, Eptifibatide wil be kept separated and mixed with the polymer solution before injection. Below trials were conducted to evaluate the effect of the physical form of Eptifibatide on overall properties of the In situ Implants. For details about the trails conducted to evaluate the impact of physical form of drug refer table no. 4.65 in the experiment part of this thesis.

Table No. 51 Analytical data for trials of Eptifibatide in situ Implant, Impact of physical form of drug.

Sr. No.	Ingredients		EP/91	EP/92D	EP/93L	EP/94E	EP/95A	EP/97
	Drug form		In solvent	As such	Lyophilise	Lyophilise	Lyophilise	Lyophilise
1	Assay (%w/w)		97.4	98.5	97.3	98.2	96.3	96.7
2	Appearance		White	White	White	White	White	White
3	In vitro release	1 Hr	3. 91	2.51	5.51	1.14	4.11	9.21
		8 Hrs.	7. 89	7.82	10.10	3.61	9.11	21.34
		12 Hrs.	21. 34	18.14	24.44	10.31	24.44	29.42
		24 Hrs.	28. 33	25.05	30.65	25.10	30.65	44.15
		48 Hrs.	59. 45	40.22	65.34	49.14	65.34	72.04
		72 Hrs.	73. 12	68.10	78.10	68.13	78.10	83.90

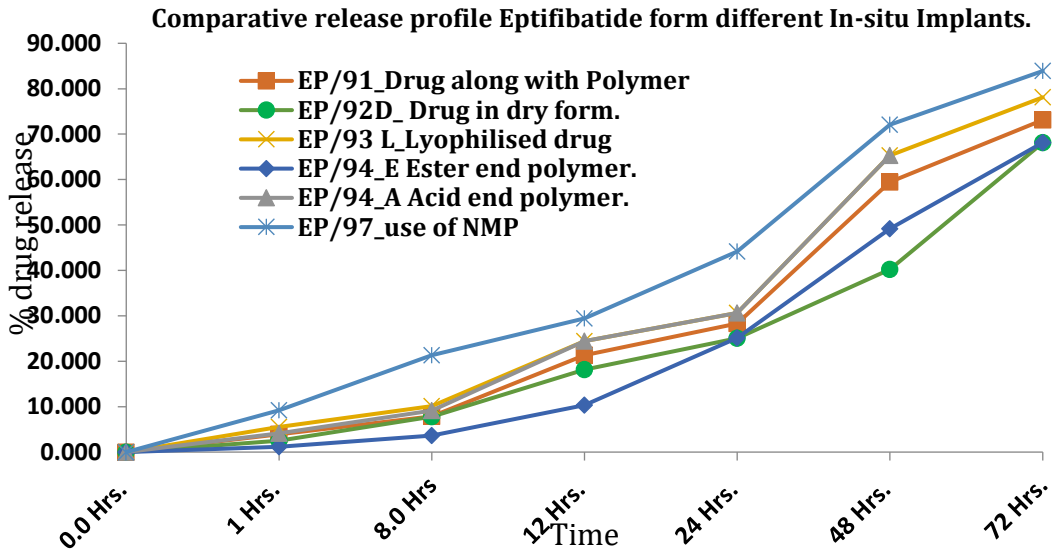

Figure No.18 Comparative release profile of Eptifibatide from different in situ implants.

The lyophilized drug gives better release when compared to the dry form of the drug. This might be due to change in the crystals structure of the drug and also due to easy wetting and solubilisation with the polymer solvent post lyophilization. Use of ester end group polymer against acid end group polymer clearly showed the difference in the degradation rate of both the polymers and thus the overall release profile, ester end polymer degrades at slower rate than acid end polymer as is observed form the release profile. Release of Eptifibatide was much faster when NMP was used as solvent to dissolve the polymer vs when DMSO was used.

Composition of optimised Eptifibatide In situ Implant

Based on the trials conducted the qualitative and quantitative composition of Eptifibatide in situ implant was finalized.

Table 53 Composition for optimised Eptifibatide in situ implant

Sr. No.	Ingredients	Role	mg/vial	%w/w
1	Eptifibatide	Drug	12.96	1.21
2	PLGA 75:25 Polymer	Biodegradable polymer	26.04	2.44
3	2-N methyl pyrolidone (NMP).	Solvent for Polymer	1.03 g	96.35
Total weight of in situ Implant			1.069 g	

Density of NMP is 1.03 g/ml.

The optimised batch was then charged on stability as per ICH guidelines and found to be stable.

10. DEVELOPMENT OF EPTIFIBATIDE NANOPARTICLES

To fulfil one of the objectives of this work was to development PLGA Nanoparticles with inclusion of methoxy polyethylene glycol phospholipid m-PEG. Here the impact of surfactant and the use of high speed during homogenisation was evaluated to get the nanoparticle of desired properties.

Table No. 54 Details of trials conducted for development of Eptifibatide nanoparticles.

Sr. No.	Ingredients	mg/vial	EP/55	EP/108	EP/120	EP/121
	Polymer: mPEG ratio		**1:0.5**	**1:1**	**1:1**	**1:1**
1	Eptifibatide	12.96	0.13	0.13	0.13	0.13
2	DMFA (ml)	--	1	1	0.6	0.6
3	PLGA 75:25	60	0.6	0.6	0.6	0.6
4	**mPEG**	30-60	0.3	0.6	0.6	0.6
5	DCM (ml)	--	2	2	2	1.5
6	PVA	--	0.90	0.90	0.90	0.90
7	Water (ml)	--	180	180	180	180
8	D-Mannitol	7.04	0.07	0.07	0.07	0.07
9	Water (ml)	--	10	10	10	10
10	Homogenisation speed	--	900	950	1000	1000

Table No. 55 Analytical data for trials conducted to formulate nanoparticles.

Sr. No.	Ingredients		EP/55	EP/108	EP/120	EP/121
	D: Additive*		**1:0.5**	**1:1**	**1:1**	**1:1**
1	Assay (Total)		95.1	93.44	86.23	84.77
2	Zeta Potential (mV)		-3.3	-4.3	-4.5	-3.2
3	PSD D90 (nm)		375.3	376.6	310.1	360.1
4	Accelerated In vitro release (Lactic Acid Buffer pH 2.2)	0.25 Hrs	27.3	22 1	29.1	25.8
		0.5 Hrs	38.5	34.5	31.0	29.1
		1.0 Hrs.	43.3	40.1	45.7	40.8
		8.0 Hrs.	57.4	54.2	64.5	62.2
		12.0 Hrs.	67.5	62.4	78.5	75.4
		24.0 Hrs.	76.4	69.6	85.7	83. 2
		48.0 Hrs	95.2	88.9	91.2	89.2
		72.0 Hrs.	94.1	89.2	92.4	91.1

Based on the above trials nanoparticles of good physicochemical characteristics were obtained. The concentration of surfactant in the external phase and the speed of homogenisation are very much crucial to achieve nanoparticles of desired dimensions. More than 90% of drug was release in all the formulations, smaller particle size gives higher surface area and thus better release profile.

Composition of optimised Eptifibatide Nanoparticles.

Based on the trials conducted and based on optimisation studies quantitative and qualitative composition of Eptifibatide nanoparticles was finalized.

Table No. 56 Composition of optimised Eptifibatide nanoparticle.

Sr.	Ingredients	Role	mg/vial	%w/w
1	Eptifibatide	API	12.96	9.26
2	PLGA 75:25 Polymer	Biodegradable polymer	60	42.86
3	mPEG	Phospholipid	60	42.86
4	D-Mannitol	Bulking /Lyo protectant	7.04	5.03
Total weight of powder per vial			140	

The prototype batch was charged on stability and found to be be stable.

Development of diluent for reconstitution

Since two of our developed formulation are powder for injection there was a need to have a reconstitution diluent which will immediately wet the powder and form uniform suspension, also it will keep those particles uniformly suspended till the time the dose is administered. For details on trials conducted to formulate diluent refer to below table. Amount of dextrose 70 which is used as viscosity increasing agent and the amount of polysorbate 80 were optimised to achieve desired viscosity and wetting. Two salts were used to achieve desired osmolality.

Table No. 47 Details of trials conducted for development of diluent for reconstitution.

Sr. No.	**Batch No.**	Role	**EP/D1**	**EP/D2**	**EP/D3**	**EP/D4**	**EP/D5**
	Batch Size		**200 ml**				
	Rationale		**First trial**	**Dex 70 Pol 80**	**Dex 70**	**Dex 70**	**Pol 80**
	Ingredients		**% w/w**				
1	Polysorbate 80	Wetting Agt.	0.1	**0.25**	0.25	**0.25**	**0.15**
2	Dextran 70	Thickening Agt.	0.1	**0.7**	**5**	10	10
3	Sodium Chloride	Osmolality Agt.	0.9	0.9	0.9	0.9	0.9
4	Sodium dihydrogen phosphate dehydrate	Osmolality Agt.	0.045	0.1	0.1	0.1	0.045
5	Sodium Hydroxide	pH adjustment	q.s.	q.s.	q.s.	q.s.	q.s.
6	Purified water	Solvent	q.s.	q.s.	q.s.	q.s.	q.s.

Dex 70: Dextran 70, Pol 80: Polysorbate 80

Analytical data for developed batches is mentioned in the table below.

Table no. 58 Analytical data for trials conducted to formulate diluent for reconstitution.

Sr.	Batch No.	EP/D1	EP/D2	EP/D3	EP/D4	EP/D5
1	pH	6.48	7.5	7.25	7.2	6.12
2	Osmolality (mOsmol/Kg)	296	307	493	474	310
3	Viscosity (mpas)	0.937	1097	3.733	6.564	6.789
5	Appearance, reconstituted Msp	GW , IS	GW, IS	GW, IS	US	US

GW: Good wetting, IS: Immediate settling, US: Uniform suspension.

Thus, the composition of diluent was finalised, and the diluent was able to give good wetting of the microspheres and nanoparticles. Also, it kept the microspheres and nanoparticles uniformly suspended till the time they were taken in syringe during injection.

The optimised batch of diluent was charged on stability and found to be stable.

11. IN VITRO RELEASE METHOD DEVELOPMENT

In-vitro release testing is an economic and useful quality control tool to effectively assure acceptable product quality during different stages of development and production.

Since we intend to develop a controlled drug delivery system that will release the drug over a period of one month, there is a need to develop two in vitro release methods. The first is the quality control (QC) method which will help to evaluate the quality of routine batches in a very short time span. The other will be real time in vitro release method that will actually monitor the release profile of the entrapped drug for over a specified period of time.

- Accelerated in vitro release testing method (For QC and batch release)
- Real-time in vitro release testing method (To monitor the overall release profile)

Determination of drug solubility in different buffers.

The first step in the development of in vitro release method, involved determination of solubility of the drug in different buffers followed by solution state stability testing of the dissolved drug in the selected buffers.

Following buffers were chosen for solubility and solution state stability.

1. Hydrochloric Acid buffer 0.1 N pH 2.2
2. Lactic Acid Buffer pH 2.2
3. Acetate Buffer pH 4.5
4. Phosphate buffer pH 6.8
5. Phosphate buffer pH 7.4

Solution state stability of the drug in the selected buffers.

Based on the results of solubility study and reported literature [25] decided to use Acetate buffer pH 4.5, Lactic acid buffer pH 2.2, Phosphate buffer pH 6.8, and Phosphate buffer pH 7.4 for dissolution method development. A known quantity of drug solution (200 ppm) in the above buffers was prepared and then stored at two different temperatures for about 35 days. The storage temperatures selected for study were 37° C and 52° C The content of solution was estimated after 1, 2, 3, 7, 14, 21, 28 and up to 35 days.

Buffers with maximum solubility were selected and studied for stability to identify a suitable buffer. Hence, the stability for Eptifibatide was determined in the selected buffers for further method development. The selected buffers included Acetate buffer pH 4.5, Lactic acid buffer pH 2.2, Phosphate buffer pH 6.8 and Phosphate buffer pH 7.4. The storage temperature was selected with the intention to develop accelerated in vitro dissolution method at 52° C as the glass

transition temperature of the polymer is about 42-45° C. For real time release temperature of 37° C was chosen to keep in line with body temperature.

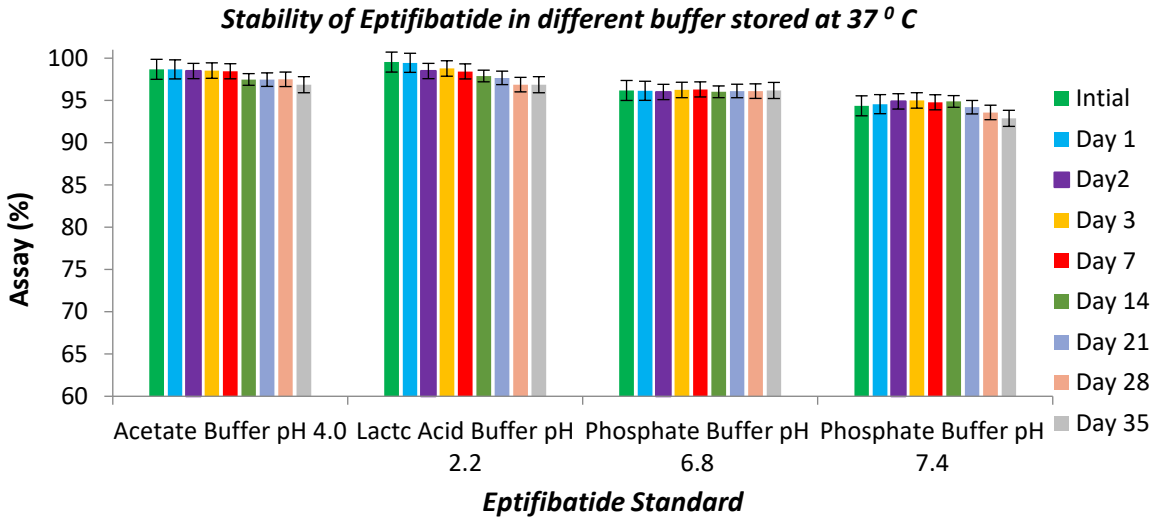

Figure No. 19 Stability of Eptifibatide in different buffer stored at 37° C

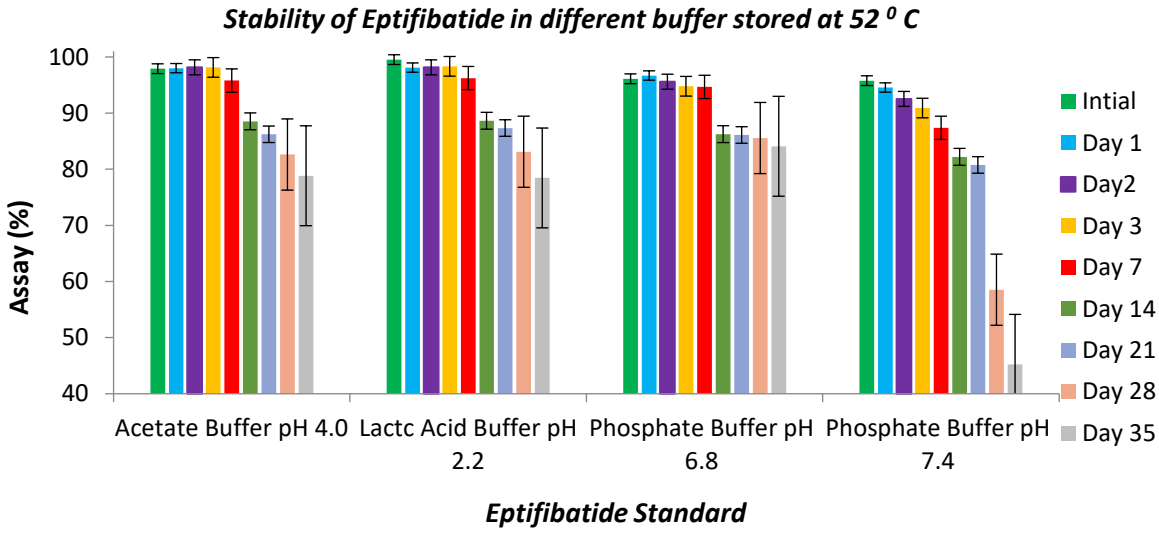

Figure No. 20 Stability of Eptifibatide in different buffer stored at 52° C

Eptifibatide was found to be stable in all the selected buffers when placed at 37° C, with less than 2% degradation from initial value and with RSD below 5%. Eptifibatide was also found to be stable up to 4 days as was desired in all buffers when stored at 52° C. Thus, based on the above results Lactic Acid buffer pH 2.2 was selected for the accelerated in vitro quality control release testing method, and Acetate buffer pH 4.5 was selected as the accelerated in vitro release discriminatory method. Phosphate buffer pH 6.8 was selected for real time in vitro release study.

Optimisation of the in vitro release testing method.

The critical component of microsphere is biodegradable polymer which is hydrophobic hence there is a need to add a wetting agent to wet this hydrophobic polymer. Hence the amount of wetting agent used was optimised. Polyvinyl alcohol was used as the wetting agent.

The shaking of the dissolution medium will have a profound impact on the release profile of the drug form the microspheres hence the impact of shaking and non-shaking apparatus was evaluated. To fasten the release profile in accelerated in vitro release the impact of temperature was evaluated. The method used to determine the percentage drug release was also evaluated.

Table No. 59 Summary of parameters evaluated for optimisation of in vitro release.

Parameter	Condition	Observation	Optimized Parameters
Selection of buffer	Lactic acid buffer pH 2.2	Better and complete release was obtained in Lactic Acid buffer pH 2.2	Lactic acid buffer pH 2.2 was selected for QC medium
	Acetate buffer pH 4.5	Gradual release was obtained in Acetate buffer pH 4.5	Acetate buffer pH 4.5 was selected as discriminatory buffer
	Phosphate buffer pH 6.8	Better and complete release was obtained in Phosphate buffer pH 6.8	Phosphate buffer pH 6.8 was chosen for real time in vitro studies.
Release temperature	37° C	Very slow and incomplete release obtained.	52°C was chosen as temperature during in vitro dissolution.
	42° C	Very slow and incomplete release obtained.	
	47° C	Very slow and incomplete release obtained.	
	52° C	Gradual and controlled release obtained	
	57° C	Fast release was obtained.	
Surfactant concentration (Polyvinyl alcohol)	0.1%, 0.2%, 0.4%, 0.5% and 1%	Very slow and incomplete release obtained at lower PVA concentrations.	0.4% and 1% was chosen for Lactic Acid & Acetate buffer. 0.5% was chosen for Phosphate buffer.
		Gradual and controlled release was seen at 0. 4 % and above concentration.	
Stirring speed of buffer medium	Shaking water bath	Gradual and complete release High shaking cause frothing	50 rpm was chosen
	Non shaking water bath	Slow and in complete release	Shaking is must.
Entrapped, Free and cumulative	Entrapped	Similar release profile seen	Cumulative
	Free	Similar release profile seen	
	Cumulative	Similar release profile seen	

12. ANIMAL STUDIES

Pharmacokinetic and pharmacodynamic studies of the developed dosage form was evaluated.

Dose selection study

Before the actual study of the developed formulation in animal it was decided to check the minimum effective concentration and the specificity of the bioanalytical method.

Animal dose calculation for Eptifibatide

Human dose: 180 µg /kg iv bolus & then 2µg /kg/min up to 96 hours

Therefore 0.18 mg/kg

AED (mg/kg) = Human dose (mg/kg) X Km ratio

Km ratio = Body weight (kg) / Body surface area (m^2)

Rat weight: 150 grams, i.e., 0.15 kg

Rat body surface area: 0.025 m^2

Rat Km ratio = 0.15 /0.025 = 6

AED (mg/kg) = 0.18 mg/kg X 6

= 1.08 mg / rat

Thus, Eptifibatide plain injection were dosed at 1.08 mg/ rat in three male rats by iv bolus via lateral vein in the tail, the plasma profile as well as the clotting time was observed. The impact of combining an infusion dose (16 µg/min for 1 hour) immediately after the bolus dose was also evaluated.

Table No. 60 Plasma concentration of Eptifibatide after single dose of Eptifibatide.

Dose	Time	Plasma Drug (ng/ml)	Clotting time (sec)	
			Control	Inj.
1.08 mg / rat (Bolus)	0	110	65	NA
	0.25	95.3	66	310
	0.5	63.4	54	279
	1	40.1	60	250
	2	32.6	55	200
	3	25.6	53	140
	4	9.5	56	80
1.08 mg / rat (Bolus) + 16 µg/min for 1 hour	0.0	120.1	65	NA
	0.25	98.5	66	315
	0.5	80.4	54	285
	1	56.2	60	245
	2	41.5	55	180
	3	34.6	53	143
	4	21.0	56	152
	5	18.6	48	115
	6	10.5	51	95

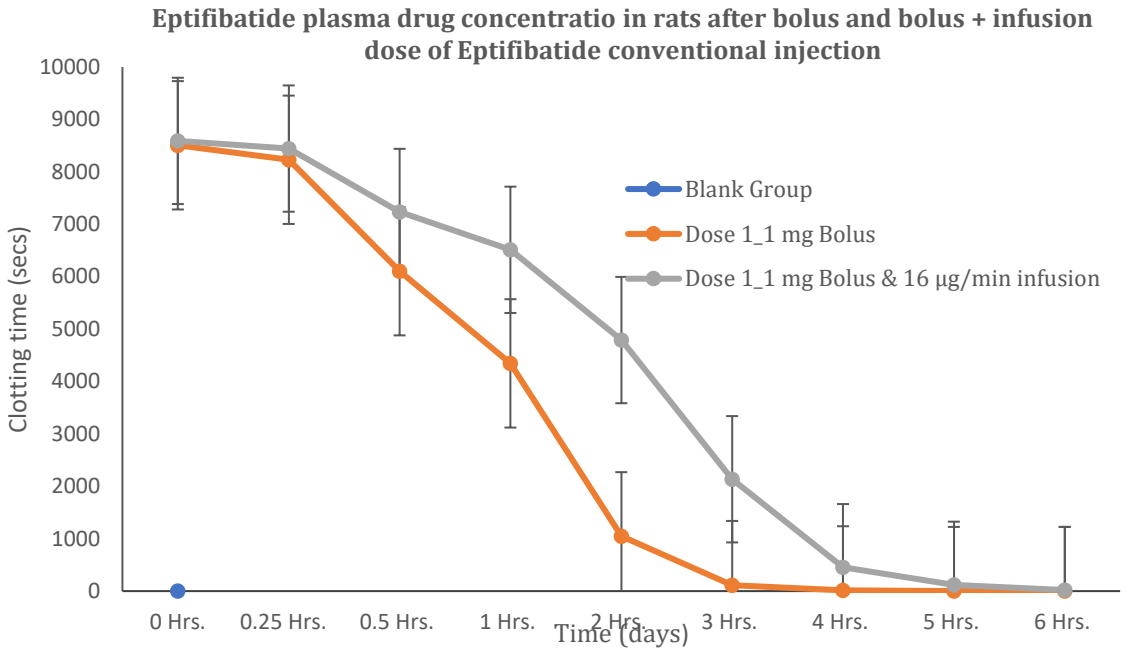

Figure No. 21 Eptifibatide plasma drug concentration after bolus and bolus with infusion

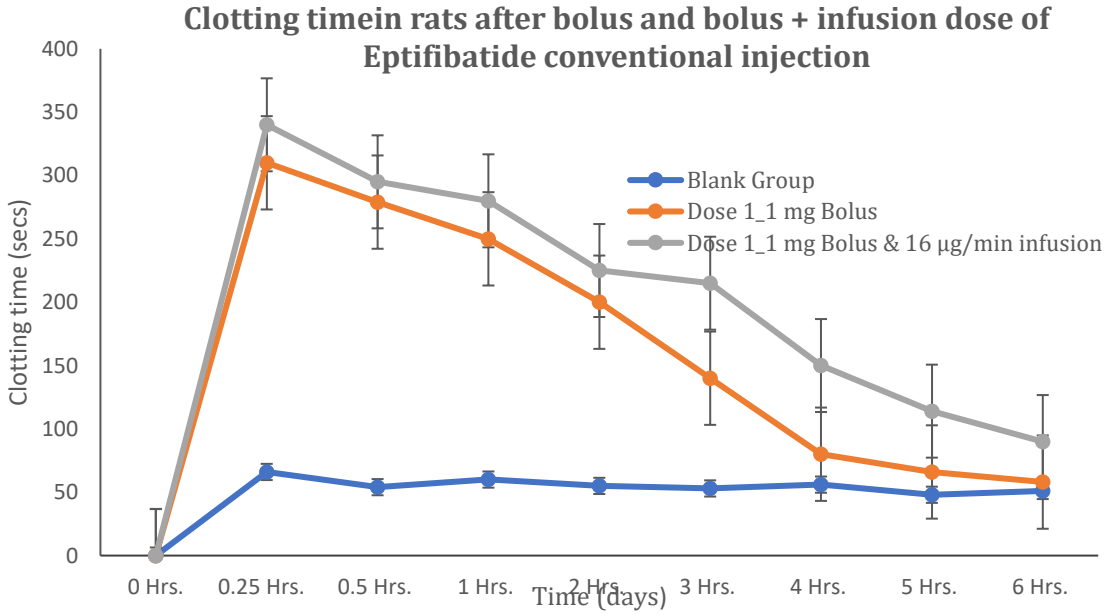

Figure No. 22 Blood clotting time after bolus and bolus with infusion

Based on the data obtained it was observed the selected dose was able to increase the clotting time in rat thus it was proved that this dose can be used for further evaluation. Also, there was drastic drop in the plasma drug concentration of the drug in around 4 hours the concentration dropped below 10 ng/ml when injected as bolus dose. Addition of infusion reduced this drop in the plasma drug concentration and the drug concentration was maintained above 10 ng/ml for another 2 hours.

Considering the limitation associated with for longer infusion time in animal, it was decided to keep infusion time to maximum 1 hour. Thus, the the dose selected for plain injection of Eptifibatide was 1.1 mg / animal bolus, followed by infusion of 16 µg/min/hr after 10 minutes of bolus injection.

Pharmacokinetic studies in animals

In vivo animal studies to evaluate the PK profile of developed Microspheres.

To evaluate the in vivo performance of the developed microspheres here as per the animal dose calculated the quantity of microspheres required for the selected dose was calculated. Now 12.96 mg of Eptifibatide is present in 80 mg, i.e., per vial. So, about 20.37 mg of microspheres were taken in a vial which was reconstituted with 3 ml of diluent to get a solution of 1.1 mg/ml. One ml each was then injected in three rats. The developed microspheres were compared with plain injection.

Study Design

Strain: Sprague Dawley Rats

Number of animals / groups: 3 rats per group, two groups of animals

Route of administration: sc for microspheres and iv for plain injection.

Observation parameters: Plasma Eptifibatide level for up to 35 days (microspheres)

Bioanalytical method: LCMS-MS

Study centre: BSV preclinical facility.

Table No. 61 Plasma concentration of Eptifibatide after single dose of Eptifibatide microspheres in rats

Plasma concentration of Eptifibatide in SD Rats (ng/ml)						
Dosage Forms		Eptifibatide Microspheres				Plain Injection
Time	Control	R1	R2	R3	Avg.	Avg.
0.5 h	0.00	70.81	98.92	89.76	86.49	80.87
1 h	0.00	78.35	105.89	106.12	96.78	56.34
2 h	0.00	65.66	97.23	117.25	93.38	41.61
3 h	0.00	20.98	35.82	31.06	29.29	34.03
4 h	0.00	10.13	14.63	31.91	18.89	21.54
6 h	0.00	4.42	5.50	10.40	6.77	10.45
8 h	0.00	3.26	4.71	5.76	4.57	0.00
24 h	0.00	4.53	5.15	4.62	4.77	0.00
2 D	0.00	3.60	4.21	3.11	3.64	0.00
4 D	0.00	0.00	2.78	2.66	1.81	0.00
7 D	0.00	0.00	2.30	2.50	1.60	0.00
14 D	0.00	1.74	1.90	1.87	1.84	0.00
21 D	0.00	1.10	1.00	0.99	1.03	0.00
28 D	0.00	0.62	0.56	0.45	0.54	0.00
35 D	0.00	0.01	0.023	0.012	0.02	0.00

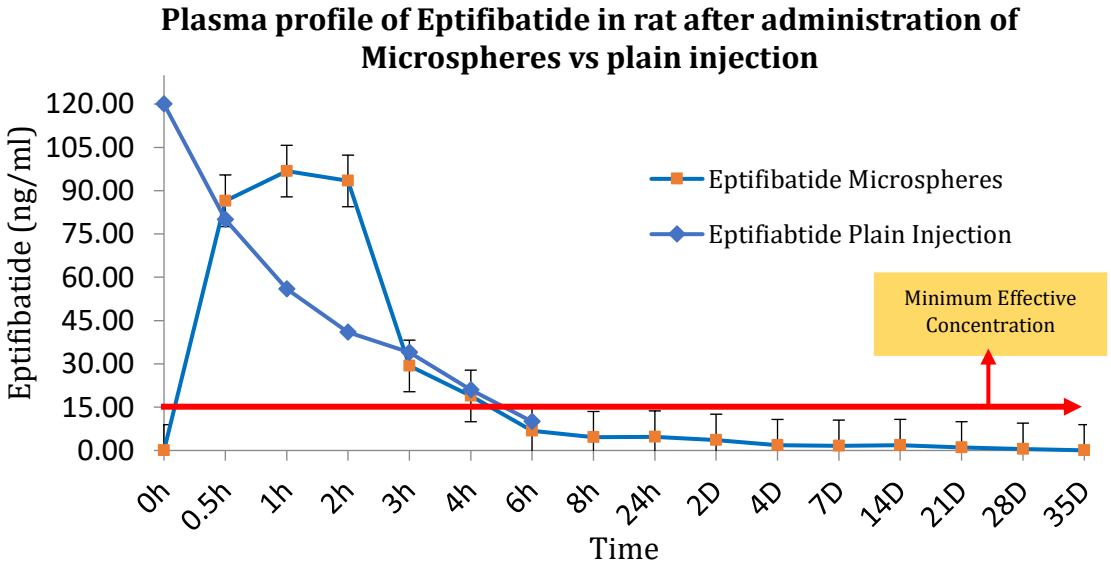

Figure No. 23 PK profile of Eptifibatide microspheres in SD rats

The developed microspheres formulation was able achieve Cmax immediate within half hour due to the presence of surface free drug. The Cmax was like the plain injection, and it was also able to maintain the drug concentration in the plasma for up to 28 days (0.5 ng/ml). However, concentration below 15 ng/ml, that was decided as minimum effective concentration for these studies dropped after 6 hours this was due the lag phase usually observed with biodegradable polymer-based microspheres. By the time the polymer got

hydrated the plasma concentration dropped. Thus, it clearly shows a need to have an inclusion of additives or adopting a process which will reduce the free drug and loosely bound drug on the surface of the microspheres. There is also a need to reduce the lag phase of polymer hydration so that the required concentration can be maintained.

In vivo animal studies to evaluate the PK profile of developed Implants.

To evaluate the in vivo performance of the developed Implants Batch no. EP/70 was injected in Sprague Dawley rats. The quantity of implant required for the selected dose was calculated. Now 12.96 mg of Eptifibatide is present in 52 mg, i.e., per implant. So, implant weighing 4 to 6 mg were cut and then injected in rats using 16-gauge needle. The developed implants were compared with plain injection.

Study Design

Strain: Sprague Dawley Rats

Number of animals / groups: 3 rats per group, two groups of animals

Route of administration: sc for implants and iv for plain injection

Observation parameters: Plasma Eptifibatide level for up to 35 days (Implants)

Bioanalytical method: LCMS-MS

Study Centre: BSV preclinical facility.

Table No. 62 Plasma concentration of Eptifibatide after single dose of Eptifibatide implant in rats

Plasma concentration of Eptifibatide in SD Rats (ng/ml)						
Dosage Forms		**Eptifibatide Implant**				**Plain Injection**
Time	**Control**	**R1**	**R2**	**R3**	**Avg.**	**Avg.**
0.5 h	0.00	60.23	58.40	59.11	59.25	80.87
1 h	0.00	65.44	67.50	66.59	66.51	56.34
2 h	0.00	80.49	82.10	79.11	80.57	41.61
3 h	0.00	90.13	91.33	90.12	90.53	34.03
4 h	0.00	85.10	86.11	86.49	85.90	21.54
6 h	0.00	74.29	73.92	74.99	74.40	10.45
8 h	0.00	65.64	66.29	67.33	66.42	0.00
24 h	0.00	20.33	21.34	22.35	21.34	0.00
2 D	0.00	18.39	17.69	17.58	17.89	0.00
4 D	0.00	14.30	15.44	14.56	14.77	0.00
7 D	0.00	10.40	9.99	10.45	10.28	0.00
14 D	0.00	7.29	7.44	7.59	7.44	0.00
21 D	0.00	5.11	5.78	5.61	5.50	0.00
28 D	0.00	3.440	2.990	3.120	3.18	0.00
35 D	0.00	1.330	1.400	1.450	1.39	0.00

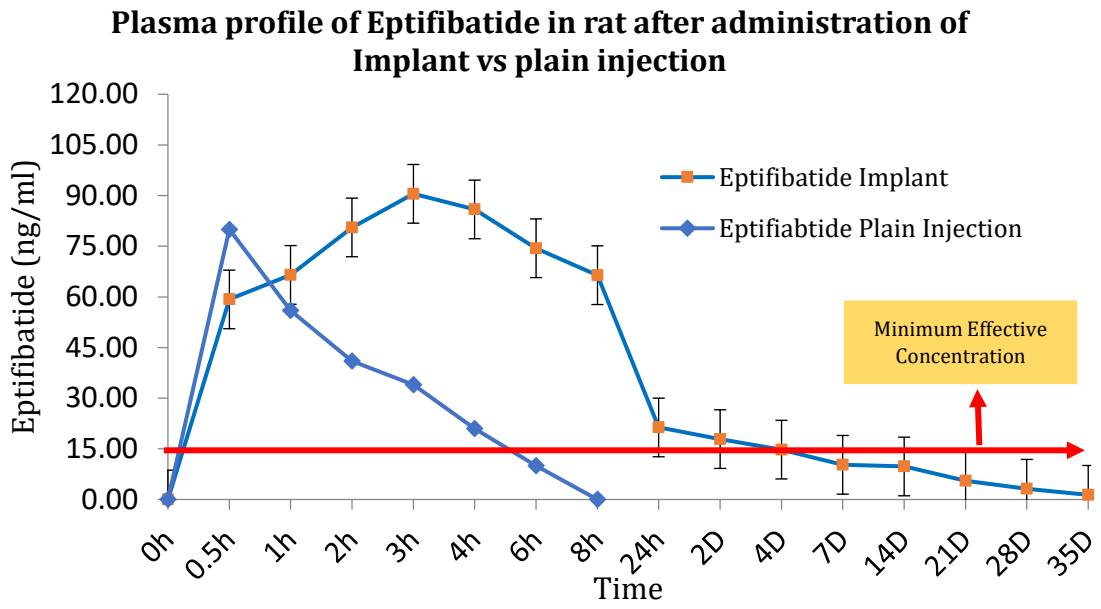

Figure No. 24 PK Profile of Eptifibatide implant in SD rats.

The developed implant formulation was able achieve Cmax after three hours possibly suggesting the impact of the implant dimension and time required for hydration. As was observed with microspheres, implant don't show burst release and it was able to maintain the drug concentration up to 15 ng/ml by seventh day. Thus, the developed implant is releasing the drug at a faster rate suggesting a need to use polymer of higher inherent viscosity/ molecular weight. The dimension of the implant is also very crucial as it decide the hydration time.

In vivo animal studies to evaluate the PK profile of developed In situ implant.

To evaluate the in vivo performance of the developed in situ implants Batch no. EP/97 was injected in Sprague Dawley rats. Tthe quantity of in situ implant required for the selected dose was calculated. Now 12.96 mg of Eptifibatide is present in 554 mg, i.e., per vial. Therefore, to inject 1.1 mg per rat proportionate amount of drug polymer and NMP were mixed (141 mg) and then divided in three parts and injected into rats.

Study Design

Strain: Sprague Dawley Rats

Number of animals/groups: 3 rats per group, two groups of animals.

Route of administration: sc for in situ implant and iv for plain injection.

Observation parameters: Plasma Eptifibatide level for up to 35 days (in situ implants)

Bioanalytical method: LCMS-MS

Study Centre: BSV preclinical Facility.

Table No. 63 Plasma concentration of Eptifibatide after single dose of Eptifibatide In situ implant in rats

Plasma concentration of Eptifibatide in SD Rats (ng/ml)						
Dosage Forms		Eptifibatide In situ Implant				Plain Injection
Time	Control	R1	R2	R3	Avg.	Avg.
05 h	0.00	20.44	23.14	24.33	22.64	80.87
1 h	0.00	65.44	67.50	66.59	66.51	56.34
2 h	0.00	34.57	32.44	33.54	33.52	41.61
3 h	0.00	18.88	18.43	18.43	18.58	34.03
4 h	0.00	16.99	17.01	17.00	17.00	21.54
6 h	0.00	34.66	32.99	33.00	33.55	10.45
8 h	0.00	60.32	61.10	60.30	60.57	0.00
24 h	0.00	45.44	44.56	43.50	44.50	0.00
2 D	0.00	25.40	25.32	25.11	25.28	0.00
4 D	0.00	22.45	22.13	22.55	22.38	0.00
7 D	0.00	21.45	20.44	21.34	20.67	0.00
14 D	0.00	18.01	18.90	18.95	18.03	0.00
21 D	0.00	16.12	17.13	15.44	16.27	0.00
28 D	0.00	7.010	8.32	6.44	7.01	0.00
35 D	0.00	3.010	3.24	3.10	3.13	0.00

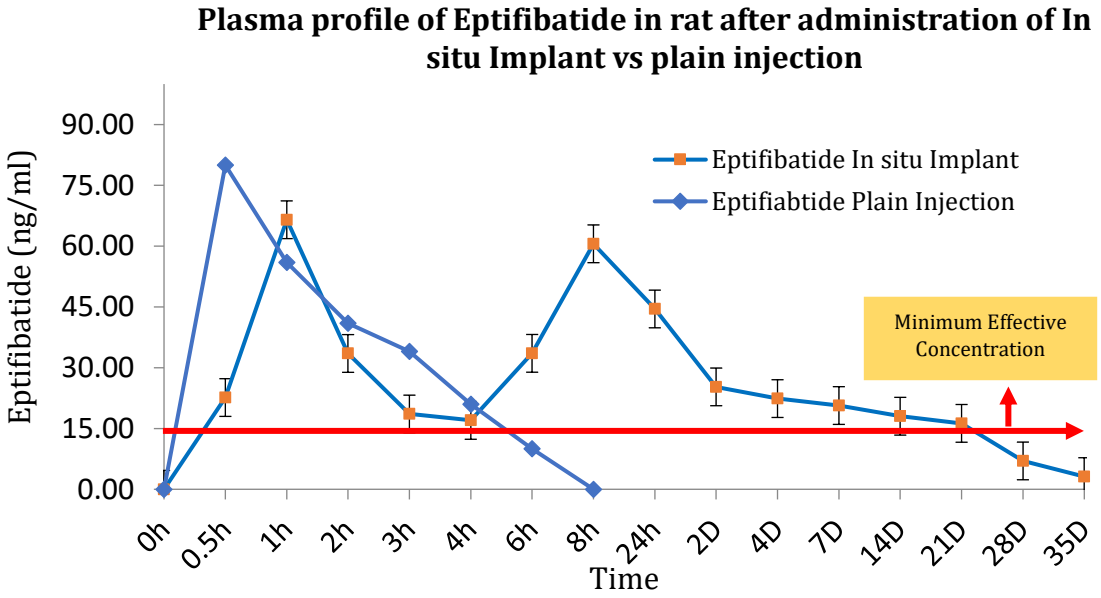

Figure No. 26 PK Profile of Eptifibatide In situ Implant in SD Rats.

The developed in situ implant gave initial burst in one hour possibly due to unentrapped drug. After this initial burst a lag phase was observed for up to three hours this was basically due to the formation of in situ implant. Here since there is no control on the shape of the implant formed inside the body, the lag phase will vary depending upon the site of administration and

the forced used during the injection. After the lag phase the in-situ implant was again able to achieve drug concentration close to Cmax and was able to maintain the drug concentration above the minimum effective concentration for up to twenty-fifth day. Thus, the developed formulation provided a controlled release of the entrapped drug which can be helpful over conventional injection.

In vivo animal studies to evaluate the PK profile of developed Nanoparticles.

To evaluate the in vivo performance of the developed Implants Batch no. EP/121 was injected in Sprague Dawley rats. The quantity of nanoparticles required for the selected dose was calculated. Now 12.96 mg of Eptifibatide is present in 140 mg, i.e., per vial. Therefore about 35.64 mg of nanoparticles were taken in a vial which was then reconstituted with 3 ml of diluent to get a solution of 1.1 mg/ml. So, 1 ml each was then injected in three rats.

Study Design

Strain: Sprague Dawley Rats

Number of animals/groups: 3 rats per group, two groups of animals

Route of administration: sc for nanoparticles and iv for plain injections.

Observation parameters: Plasma Eptifibatide level up to 35 days (nanoparticles)

Bioanalytical method: LCMS-MS

Study Centre: BSV preclinical facility.

Table No. 64 Plasma concentration of Eptifibatide after single dose of Eptifibatide Nanoparticles in rats

Plasma concentration of Eptifibatide in SD Rats (ng/ml)						
Dosage Forms		Eptifibatide Nanoparticles				Plain Injection
Time	Control	R1	R2	R3	Mean	Avg.
0.5 h	0.00	47.4	47.6	50.1	48.37	80.87
1 h	0.00	81.03	79.45	78.45	79.64	56.34
2 h	0.00	45.01	44.56	43.9	44.49	41.61
3 h	0.00	33.4	33.45	33.12	33.32	34.03
4 h	0.00	31.49	32.01	31.99	31.83	21.54
6 h	0.00	32.33	31	34.55	32.63	10.45
8 h	0.00	31.22	30.56	29.45	30.41	0.00
24 h	0.00	29.13	28.44	29.45	29.01	0.00
2 D	0.00	25.34	24.55	25.32	25.07	0.00
4 D	0.00	22.45	22.13	22.55	22.38	0.00
7 D	0.00	20.6	19.08	20.99	20.22	0.00
14 D	0.00	19.63	19.67	19.87	19.72	0.00
21 D	0.00	18.01	18.66	18.56	18.41	0.00
28 D	0.00	16.32	16.78	16.56	16.55	0.00
35 D	0.00	12.88	12.89	13.56	13.11	0.00

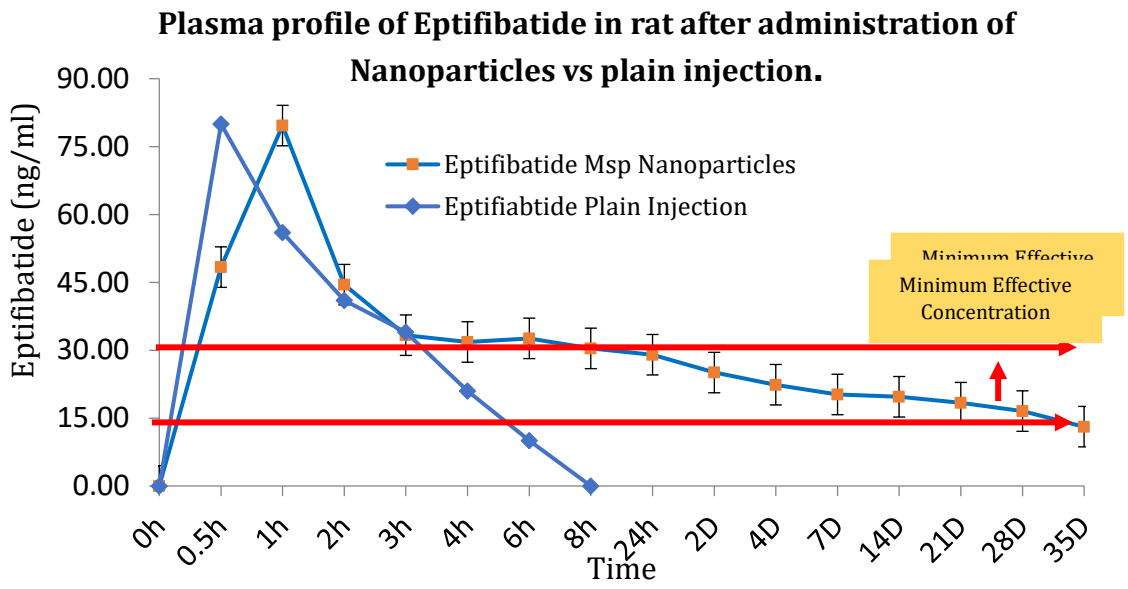

Figure No. 27 PK Profile of Eptifibatide Nanoparticles in SD Rats

The developed nanoparticles gave initial burst in around one hour this is due to the free unentrapped drug present on the surface. Also, nanoparticle provides large surface area which is beneficial for faster hydration of the polymer, and this also will give unwanted burst release. The formulation was able maintain the drug above minimum effective concentration for up to twenty-eight days this was basically due to balance achieved between polymer hydration and release of the drug, which prevented the drop of drug in plasma.

Pharmacodynamic studies in animals

Clotting time studies

To evaluate the efficacy of the developed formulation we also evaluated the clotting time in animals injected with Eptifibatide microspheres against the control animals.

Table No. 65 Blood clotting time in rat after administration of developed dosage forms.

Time Point	Clotting time (seconds)					
	Control	Microspheres	Implant	In situ Implant	Nanoparticles	Plain Injection
0.25 hr	62	286	245	290	283	310
0.5 hr	65	257	267	281	280	279
1 hr	59	241	254	278	200	250
2 hr	55	150	256	269	178	200
3 hr	58	112	245	140	160	140
4 hr	62	98	200	156	168	156
6 hr	64	95	187	145	170	112
8 hr	57	57	165	256	158	56
24 hr	49	49	140	250	154	62
2 D	55	50	128	234	160	66
4 D	65	53	94	240	163	45
7 D	53	61	85	225	158	52
14 D	44	51	90	180	120	50
21 D	51	52	52	150	110	56
28 D	78	60	55	125	90	58
35 D	39	58	60	112	60	59

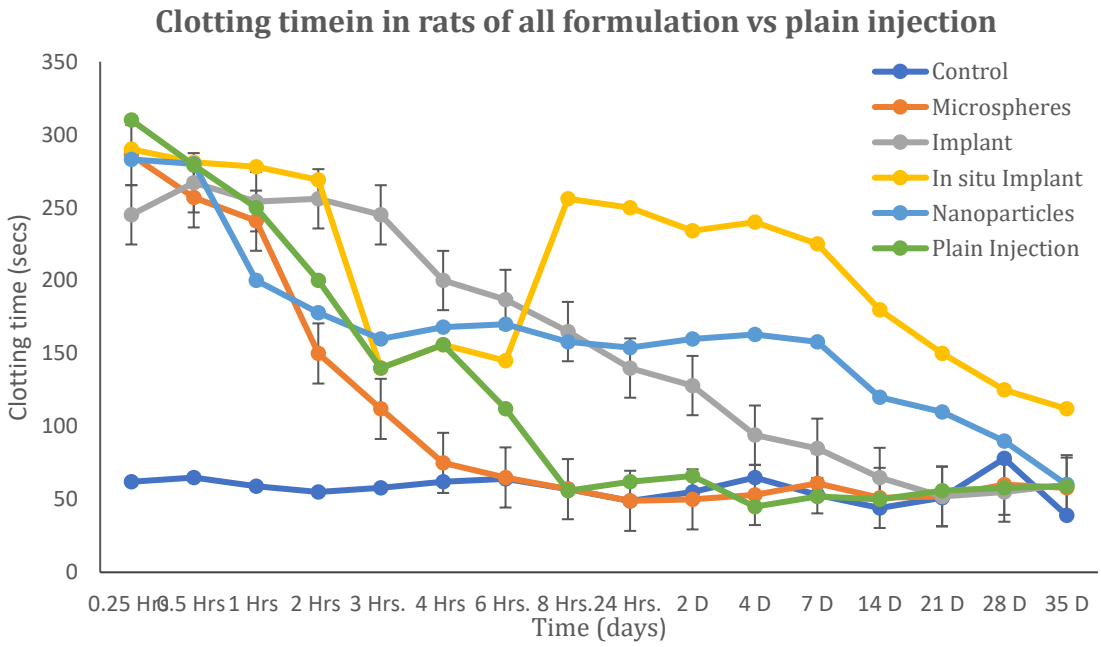

Figure No. 28 Comparative clotting time in rat.

Based on the data it is evident that formulated nanoparticles and in situ implant were able to prevent clotting of blood for up to 28 days that was desired. Whereas developed implant was able to prevent clotting time for up to 14 days and the developed microspheres were up to 6 hours. Form this is very much evident among all the developed formulations nanoparticles and in situ implant were much better in providing a controlled release for a longer period of time. As such no major difference was observed in clotting time after administering the developed dosage forms. However, this can be beneficial to prevent excessive bleeding in patients, more data needed to conclude on this.

Drug Efficacy study using Thrombus model in rats.

To reconfirm the data observed in clotting time study the promising batches were further evaluated using Thrombus model. The details for inducing thrombus and handling of animals as well as administration of the developed dosage form has been explained in section of experimental part of this thesis. Here the clotting time as well as platelet count was evaluated at specified times points the details of this is mentioned in the table below.

Table No. 66 Platelet count and clotting time observed in Thrombus model study.

Test	Time	Blank grp	Model Grp	Plain Inject.	EP Nanoparticles
Platelet count	Day 1	60010^9/l	94510^9/l	68910^9/l*	65010^9/l
Clotting time		66 sec	40 sec	250 sec	160 sec
Platelet count	Day 7	61010^9/l	92010^9/l	67310^9/l*	65610^9/l
Clotting time		69 sec	35 sec	230 sec	175 sec
Platelet count	Day 14	61210^9/l	96010^9/l	68010^9/l*	63610^9/l
Clotting time		70 sec	30 sec	225 sec	135 sec
Platelet count	Day 28	62002^9/l	88910^9/l	67110^9/l*	65010^9/l
Clotting time		60 sec	36 sec	235 sec	110 sec
Platelet count	Day 35	62110^9/l	81010^9/l	66310^9/l*	68910^9/l
Clotting time		68 sec	37 sec	230 sec	100 sec

* Plain injection was given on daily basis at dose of 1.1 mg/ animal followed by infusion of 16µ/ animal for 1 hour.

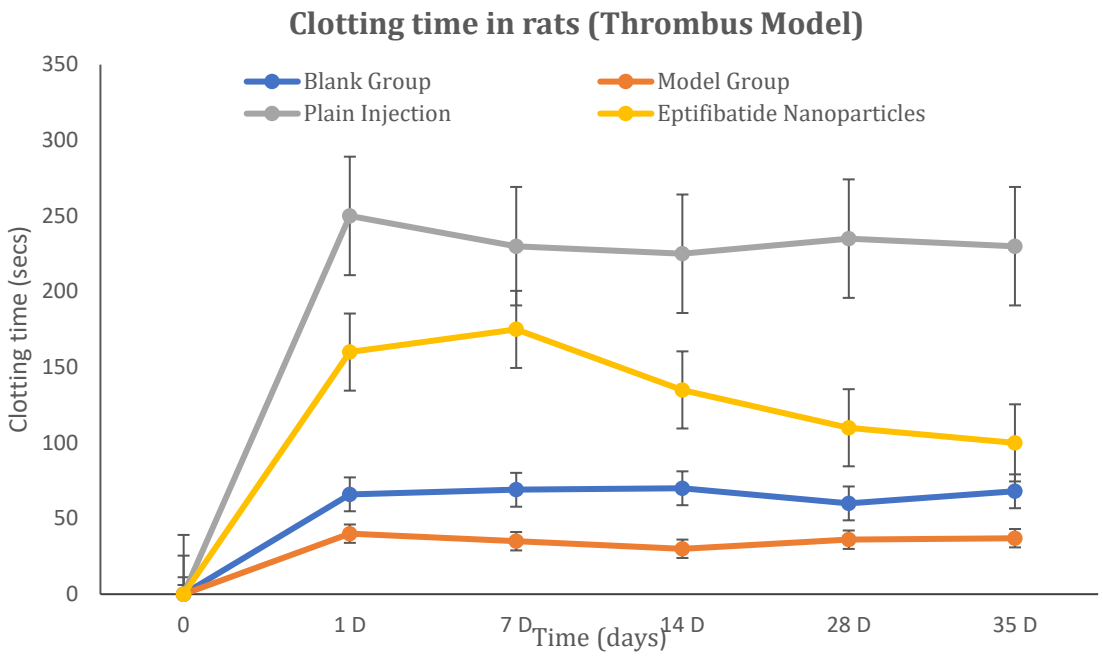

Figure No. 29 Comparative blood clotting time of different animal groups in Thrombus model study.

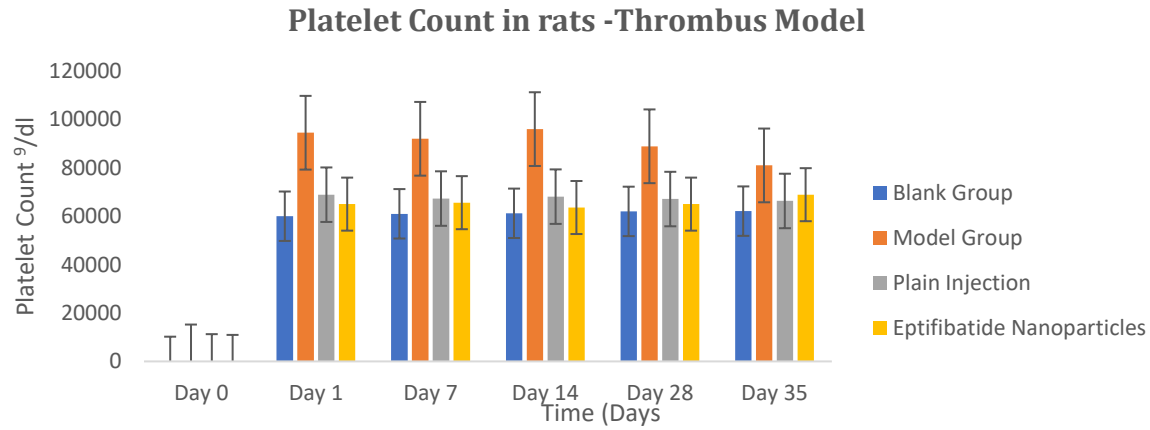

Figure No. 30 Comparative Platelet count of different animal groups in Thrombus model study.

Thus, as was observed in this study induction of thrombus was clearly seen when compared against blank group. Post administration of the drug both plain injection and developed Eptifibatide nanoparticles, there was increase in the clotting time when compared against blank and the model group clearly indicating antithrombotic action. Also, the platelet count was reduced after administration of plain injection and developed Eptifibatide nanoparticles. The efficacy level was comparable among both the formulations, however plain injection was required to be given on daily basis whereas only one depot injection was administered in case of Eptifibatide nanoparticles. Thus, the efficacy of the developed formulation was clearly observed in this study.

Acute toxicity studies

The acute toxicity study of the developed formulation was evaluated here. Initial samples as well as 3 months samples charged on stability were evaluated for toxicity studies. The clinical observations were noted for the animals for up to 14 days. The observed data in mentioned in the table below. The animals were specifically observed for any signs of bleeding.

Table No. 67 Acute toxicity studies for developed dosage forms in mice.

Test Substance	Dose (mg/mice)	Body Weight Observation	Clinical Signs	Gross Observations	Mortality %
Eptifibatide plain Injection*	4000 µg/kg Single bolus Injection	**Male-** Normal gain observed on day 7 & day 14. **Female-** Normal gain observed on day 7 & day 14.	Mild to moderate drowsiness was observed in all animals from immediately after inj. up to 6 hrs. Slight bleeding observed on day 1.	No abnormality detected in terminally sacrificed animals.	00
Eptifibatide Microspheres (Initial & 3 M sample)		**Male-** Normal gain observed on day 7 & day 14. **Female-** Normal gain observed on day 7 & day 14.	Mild to moderate drowsiness was observed in all animals from immediately after inj. up to 6 hrs. Slight bleeding observed on day 6	No abnormality detected in terminally sacrificed animals.	00
Eptifibatide Implant (Initial & 3 M sample)		**Male-** Normal gain observed on day 7 & day 14. **Female-** Normal gain observed on day 7 & day 14.	Mild to moderate drowsiness was observed in all animals from immediately after inj. up to 6 hrs. Slight bleeding observed on day 8	No abnormality detected in terminally sacrificed animals.	00
Eptifibatide In situ Implants (Initial & 3 M sample)		**Male-** Normal gain observed on day 7 & day 14. **Female**- Moderate reduction observed on day 7 normal gain on day 14.	Mild to moderate drowsiness was observed in all animals from immediately after inj. up to 6 hrs Slight bleeding observed day 8 & 14	No abnormality detected in terminally sacrificed animals.	00
Eptifibatide In situ Nanoparticles (Initial & 3 M sample)		**Male-** Normal gain observed on day 7 & day 14. **Female**- Moderate reduction observed on day 7 normal gain on day 14.	Mild to moderate drowsiness was observed in all animals from immediately after inj. up to 6 hrs Slight bleeding observed on day 5 & 12	No abnormality detected in terminally sacrificed animals.	00

***** Complete dose of plain injection was administered by IV on day one.

As such no major clinical observation was found during these 14 days acute toxicity study. Slight bleeding 1-2 ml was seen form the anus which was expected considering the know side effect and the therapeutic action of the drug. Similar observed was noted for stability samples indicating no generation of free radicals in the developed dosage form on stability. Overall, all the developed formulation were found to be safe. The developed formulation is supposed to deliver the drug for period of one month hence subacute toxicity study would have given more meaning full data however due to lack samples repeat dose toxicity study was not performed.

13. OTHER SUPPORTIVE STUDIES

To further support the development wok following supportive studies were performed.

Compatibility studies with filter used for recovery of the microspheres.

Here the compatibility of Eptifibatide microspheres with the membrane filter used during recovery and with the sterile filter used for drug filtration, was studied.

A. Membrane filter used during recovery.

During development study, cellulose nitrate filter paper was used to separate / collect the suspended microsphere form the bulk solution. The compatibility of Eptifibatide microspheres with cellulose nitrate filter paper was studied. The membrane filter was soaked in microsphere suspension after solvent evaporation stage and kept aside at controlled room temperature. The samples were collected at predetermined time points and analysed.

Table No. 68 Analytical data of membrane filter compatibility study

Batch No.	**EP/115**	**EP/115**
Test	**Initial**	**24 Hrs.**
Description	Milky white suspension	Milky white suspension
Total drug content	95.23%	95.11%
Free drug content	0.33%	0.38%
Particle size (d 50)	22.4 µm	23.5 µm
Particle size (d 90)	43.1 µm	45.4 µm
Total Impurity	1.43%	1.56%
Membrane filter	White colour	No colour change or shredding.

The filter paper did not show any physical changes at the end of the study. There was no change in assay nor any change in the particle size and impurity profile of the exposed products. Considering the time duration, the recommended contact period of the product with the filter would be limited to not more than 24 hr. Based on the observed data it was concluded that cellulose nitrate membrane disc filter is compatible with drug product.

Syringe Filters

The drug solution prepared in citrate buffer and dimethyl formamide was prepared and filtered using 0.22 µ PES syringe filter.

Table No. 69 Analytical data for drug filtration study.

Batch No.	**EP/113**	**EP/113**	**EP/114**	**EP/114**
Buffer / Solvent	**Dimethyl formamide**		**0.025 Citrate buffer**	
Test	**Before filtration**	**After filtration**	**Before filtration**	**After filtration**
Eptifibatide content (mg/ml)	1.1 mg/ml	1.0 mg/ml	1.2 mg/ml	1.1 mg/ml
Total Imp	1.01%	1.1%	0.99%	1.0%

There was no change in the drug content after filtration in the both the solvents used, thus no binding of the drug on the filter was seen, as is usually reported while filtering peptides. There was no change in the impurities thus no incompatibility was observed. As the time duration of contact of the drug solution with the filter was very short there was minimal possibility of any interaction. Hence it can be concluded that the PES syringe filter can be used for sterile filtration of the drug.

Co**mpatibility studies with silicon tubing using during manufacturing**.

Silicon tubing is often used in pharmaceutical manufacturing in transfer process such as form tank-to-tank or from tank-to-filling machine. Since the tubing has direct contact with the product, it is imperative to establish the impact of tubing on the product and that it should not interact or adsorb the drug or excipients present in the formulation.

Table No. 70 Analytical data of silicon tubing compatibility study.

Test	**Initial**	**Static Compatibility**		**Dynamic Compatibility**	
		8 Hours	**12 Hours**	**8 Hours**	**12 Hours**
Description	Milky white suspension	Milky white suspension	Milky white suspension	Milky white suspension	Milky white suspension
Total drug %	93.2%	92.8%	92.5%	93.1%	92.9%
Free drug %	0.3%	0.3%	0.4%	0.3%	0.5%
Total Imp %	1.24%	1.22%	1.23%	1.31%	1.32%

There was no change in drug content when compared with initial samples. No significant change in impurity was observed under both the condition. Thus, the tubing selected was found to be compatible with the product for up to 12 hours.

Hold time and Compatibility studies with SS manufacturing vessels.

Here the interaction of stainless steel containers / vessels used during manufacturing of Eptifibatide microspheres was evaluated by keeping suspended microsphere about 90 ml in S.S. 316 container for 8 to 24 hours at room temperature. Microsphere were recovered after the said time than lyophilized and then analysed for drug content, and other parameters. This study also helped us to evaluate the hold time up to which the product can be hold at certain stages of manufacturing without affecting the CQAs of the drug product.

Table No. 71 Analytical data for hold time and compatibility study SS Vessel.

Test	EP/117A	EP/117B	EP/117C	EP/117D
	Initial	After 8 Hours	After 24 Hours	After 24 hours
Appearance	Milky white suspension	Milky white suspension	Milky white suspension	Milky white suspension
Total drug%	94.3%	94.1%	93.4%	93.8%
Free drug %	0.2%	0.2%	0.3%	0.3%
Total Imp.%	1.3%	1.4%	1.4%	1.4%

No significant change in assay and impurity was observed in stainless steel container (S.S. 316 L) for up to 24 hours. This also indicated that the product can be hold up to 24 hours at this stage if at all there arise a need to hold the product at this stage during commercial batch manufacturing.

Container closure selection studies

A container closure system refers to the sum of packaging components that together contain and protect the dosage form. Choice of materials for primary packaging was decided based on internal studies and affordability and intended use. The container closure configuration selected for developed Eptifibatide dosages forms were based on the following criteria.

- Intended use of the drug product

-Container closure compatibility

Eptifibatide microspheres and nanoparticles are lyophilized powders for injectable suspension. These are to be reconstituted before injection hence to inspect the reconstituted suspension a clear flint glass vial is necessary. Hence it was thought to use flint type I tubular glass vials, bunged Bromo butyl rubber bungs to prevent ingress of moisture. For developed implants and in situ implants there was a need to evaluate them in a syringe for easy shelf storage and during administration. However due to the unavailability of the required syringe and to generate the

stability data both these dosage forms were studied in same USP Type I glass vials. For this study simple carton boxes were used to pack the glass vials and load them on stability.

Table No. 72 Primary packing material for the developed formulations of Eptifibatide.

Eptifibatide Microspheres	Eptifibatide Implants	Eptifibatide In situ Implants	Eptifibatide Nanoparticles
Vial & rubber bungs	Vial & rubber bungs	Vial & rubber bungs	Vial & rubber bungs
Vials	5 ml flint Tubular Ready to use (RTU) USP Type-I glass vial supplied by Schott Kaisha.		
Rubber bungs	20mm Grey bromo butyl ready to use rubber stopper(RTU), supplied by Datwyler.		
Seals	20mm Aluminum flip off orange color seal supplied by West		

Lyophilisation Cycle development

Eptifibatide is a water-soluble peptide which is entrapped inside water insoluble hydrophobic Biodegradable polymer. Formulation and entrapment of this peptide inside these biodegradable polymers is achieved using solvent evaporation techniques which gives a suspension of microspheres which is then filtered and then dried under vacuum in desiccator. Drying under desiccator takes long time and gives microsphere which are slightly hard and difficult to resuspend during reconstitution. The best alternative way is to lyophilize the collected microsphere along with bulking agent like D-mannitol. The details of thelyophilization cycle development and the final recipe is mentioned below.

DSC Analytical studies

DSC analysis of the individual component of the product was done along with the mixture of components to get the glass transition temperature of all the components. These data were used to finalize the maximum freezing temperature during freezing stage and maximum drying temperature during primary and secondary drying stage of the lyophilisation process.

Considering the DSC data freezing point and eutectic melt temperature the initial set temperature during freezing was selected as -45º C with a rate of temperature change of 0.4º C / min and hold of 120 minutes to achieve the uniform temperature across the product. The final freezing temperature was proposed as -45º C to ensure the complete freezing of the drug product. During the primary drying a cautious approach was selected as the initial shelf temperature was proposed as -30 ºC and then gradually taken to 10 ºC for the primary drying. Secondary drying was done at 40 ºC.

The selection and finalisation of the lyophilization cycle was based on below points.

- Properly dried powder was obtained.
- The moisture content of the powder was below 2 %

Thus, after initial trials below mentioned lyophilisation cycle was finalised.

Table No. 73 Final lyophilization cycle used for Eptifibatide microspheres.

Steps	**1**	**2**	**3**	**4**
Stage	**FREEZING**			
Temperature (°C)	-40	-40	--	--
Time (min)	120	180	--	--
Vacuum (MTorr)	--	--	--	--
Stage	**PRIMARY DRYING**			
Temperature (°C)	-30	-30	10	10
Time (min)	120	60	120	240
Vacuum (MTorr)	100	100	100	100
Stage	**SECONDARY DRYING**			
Temperature (°C)	40	--	--	--
Time (min)	1860	--	--	--
Vacuum (MTorr)	50	--	--	--
Final Temp	25	--	--	--

Thus, a cycle of 45 hours duration was finalised to lyophilise Eptifibatide microspheres and nanoparticles.

Gamma dose selection studies

Eptifibatide microspheres and the other developed dosage forms are intended to be used parenterally, being a parenteral product the basic requirement to be fulfilled is its sterility There are different methods for terminal sterilisation like autoclave or radiation sterilisation, however the use of either of this method is governed by the type of the product to be sterilized. The formulation of Eptifibatide Microspheres is based on microsphere technology. Here Eptifibatide is dispersed in biodegradable polymer-based matrix, in the form of microspheres (spherical shape having size of few microns). The key component of the formulation being the peptide and the polymer, use of autoclaving as a terminal sterilisation method would completely degrade the peptide along with the polymer which has low glass temperature, hence the best way to sterilise the product being radiation sterilisation. To make Eptifibatide microspheres sterile, terminal sterilisation by gamma irradiation was used. To the fix the right dose of gamma

irradiation few batches of Eptifibatide microspheres were exposed to different gamma doses and then evaluated.

Table No. 74 Analytical data for gamma dose selection study.

Gamma Dose	Before Gamma	7.5 kGy	15 kGy	25 kGy
Batch No	**EP/118**	**EP/118-7.5**	**EP/188-10**	**EP/188-25**
Total drug %	95.3%	92.4%	88.6%	85.7%
Free drug%	0.04	0.10	0.10	0.09
Particle size d 50	11.2	12.3	10.9	11.6
Particle size d 90	28.9	29.2	29.0	29.1
Total Impurity	1.34	2.19	2.45	3.21
8 Hrs	20.04	29.81	23.56	24.84
12 Hrs.	45.79	58.92	55.95	64.40
24 Hrs.	62.26	85.90	81.83	84.30
48 Hrs	80.88	93.90	90.06	91.25
Mw	10474	8256	8406	8321
Mn	5651	6407	6250	6174
PDI	1.85	1.33	1.34	1.34
Sterility	Non-sterile	Passes	Passes	Passes

The data from the study showed that there was drastic drop in drug content when the product was exposed to gamma radiation of 25 & 15 kGy. There was also an increase in the impurities as these higher doses. Increased in the radiation doses decreases the molecular weight of the polymer thus further translates into faster release profile. decreases. Slight drop in drug content was observed at a dose of 7.5kGy Although all the doses gave a sterile product, considering the increase in the impurity level, drug loss and loss of molecular mass intermediate dose of 7.5 kGy was fixed for sterilization.

Sterility

Since manufacturing the product in an aseptically requires use of sterile starting material PLGA is stored at –16 °C to –20 °C to preserve its microbiological integrity. Even though we dint had sterile starting material and even we dint had facility to manufacture the product aseptically we thought of using other alternatives where we could at least be assured that the product wont fail sterility.

Following precautions were adopted to have a product with low bioburden.

- The microspheres were manufactured in controlled condition. The bioburden will be controlled during manufacturing.

- The finished product is sterilized by gamma irradiation.
- The glass vials and the rubber bungs used for finished product were pre-sterilized and ready to use. Before starting manufacture of the product, the entire manufacturing system, i.e., manufacturing vessels, Homogeniser and SS vessels are cleaned properly and the rinsing's subjected to Endotoxin limit test.
- Water for Injection used in manufacturing is filtered through 0.2µ sterilizing grade filter and is tested for BET testing.
- The entire manufacturing is carried out in a closed system wherein the product exposure to the environment is minimum, the entire manufacturing is carried out in a controlled grade B area under the LAF.

Table No. 75 Bioburden estimation of the raw material used in the manufacturing of Eptifibatide microspheres.

Ingredients	Microbiological Quality					
	Total viable Count	**Yeast and Moulds**	**E Coli**	**Salmonella**	**S Aureus**	**P Aeruginosa**
Eptifibatide	< 200 CFU/g	< 20CFU/g	Absent	Absent	Absent	Absent
PLGA	< 10 CFU/g	< 10 CFU/g	Absent	Absent	Absent	Absent
D-Mannitol	< 20 CFU/g	< 20 CFU/g	Absent	Absent	Absent	Absent

Admixture studies

As per ICH Q8 guideline, the compatibility of the drug product with reconstitution diluent should cover the recommended in-house shelf life, at the recommended storage temperature and at the likely extreme of concentration. During clinical administration, the drug admixture may come into contact with the following components: Syringes—In cases where the infusion volume may be small the dose may be administered through a syringe pump (e.g., pediatric patients). "ICH Harmonized Tripartite Guideline—Pharmaceutical Development Q8" states the following: "The compatibility of the drug product with reconstitution diluents (e g., precipitation, stability) should be addressed to provide appropriate and supportive information for the labelling. This information should cover the recommended in- use shelf life, at the recommended storage temperature and at the likely extremes of concentration. For lyophilized product the stability of the reconstituted product needs to be evaluated.

Table No. 76 Analytical data for admixture and Compatibility study.

Condition	**Room Temperature (Batch No. EP/120)**					
Test	**Initial**		**After 4 Hrs.**		**After 8 Hrs.**	
Container/ Device	**Vial**	**Syringe**	**Vial**	**Syringe**	**Vial**	**Syringe**
Description	MWS	MWS	MWS	MWS	MWS	MWS
Total drug %	95.1	95.2	95.4	95.5	95.1	94.9
Free drug %	0.3	0.3	0.2	0.3	0.4	0.4
Particle size d 50	22.1	23.3	21.5	22.4	22.5	22.4
Particle size d 90	43.5	44.6	42.4	43.1	43.1	44.1
pH	6.72	6.74	6.81	6.91	6.73	7.10
Total Impurity	1.4	1.5	1.5	1.3	1.5	1.4

MWS: milky white uniform suspension

Based on the results obtained there in no change in drug product CQA's thus it can be concluded that the product is compatibility with the syringes and the diluent used during injection for up to 8 Hrs. However, is it advisable to use the reconstituted product immediately and discard the unused product.

Syringibility and injectability study.

It measures the ease with which the product can be withdrawn from the vial (syringibility) and the ease with which it can be injected into human being (injectability). Considering the particle size of the developed microspheres 22-gauge needle was found to be suitable. Using this 22-gauge needle attached to a syringe the entire product was withdrawn from the vial and then emptied in another empty vial. Here the entire product got easily withdrawn and with ease the entire product was emptied thus proving that the product can be easily injected in patients using 22-gauge needle.

14. CONCLUSION

Thus, based on the trials conducted following conclusion can be drawn and can use used when developing biodegradable polymer-based microspheres.

o The use of a solvent to solubilize the drug is needed for better drug loading.

o The drug should have some bonding with the polymer to have high drug loading if a suitable solvent for the drug is not available.

o Saturating the external phase is a good option but can't be used alone as such needs to be combined with approaches to have better drug loading.

o Polymer inherent viscosity is crucial and the higher the viscosity better will the drug loading however high viscosity causes high molecular weight and thus might impact the in vitrorelease profile. A proper balance needs to be achieved between the desired drug load and in vitro release.

o The inclusion of additives like polyvinyl alcohol and polyethylene glycol 400, helped reduce the lag phase (time taken for polymer for wetting). The inclusion of HP-β-CD controlled the initial unwanted burst effect whereas the presence of mPEG gave an overall controlled release. Thus, having an additive in the formulation is a good viable option available to increase the drug load and to address other challenges involved like unwanted burst release, reducing the lag phase, and stabilizing the drug during and after manufacturing.

o Among the different manufacturing methods used single emulsion solvent evaporation method is simple and easy to scale up. It gave microspheres of desired quality attributes.

o Polymer type and inherent viscosity play important role in the overall physicochemical properties of the developed microspheres. Combining two polymers in different ratios can be a good option to get the desired release profile.

o Other alternative dosage forms even though were found to be comparable to microspheres still below points need to be taken care of during development.

➢ Implants are a good alternative to microspheres however the physical form of the drug and temperature during extrusion plays a crucial role in controlling the overall parameters.

➢ In situ Implant, here also the physical form of the drug in the polymer phase plays an important role in the overall release profile. Controlling the size/shape of the implant formed after injection is important (Injection volume and pressure during injection are important)

➢ Nanoparticles provide a better alternative to microspheres considering these can be further used to target the drug at the desired site of action. The type of polymer used, and the

manufacturing method employed can have a profound impact on the physicochemical properties of the dosage forms.

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RESEARCH OUTCOME

Type of Article	Title of Article	Name of Journal & Impact Factor	Status of Publication
Review	Biodegradable Polymer-Based Microspheres for Depot Injection-Industry Perception.	Recent Advances in Drug Delivery and Formulations. Impact factor: 1.6	**Published**
Research	Preparation and Evaluation of Eptifibatide Microspheres		Under drafting
Research	Effect of inclusion of additives on physicochemical properties of Eptifibatide Microspheres		Under drafting
Research	Effect of Polymer blending on the physicochemical properties of Eptifibatide Microspheres		Under drafting

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